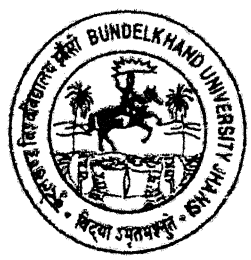
Microsatellite enriched genomic library construction, isolation and characterization of microsatellite DNA markers in *Chitala chitala* for population genetics studies.

Thesis

Submitted to
Bundelkhand University, Jhansi (U.P.)



For the degree of

DOCTOR OF PHILOSOPHY

In

Biotechnology

By

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(2008)

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CERTIFICATE

This is to certify that the work entitled **"Microsatellite enriched genomic library construction, isolation and characterization of microsatellite DNA markers in *Chitala chitala* for population genetics studies"** is a piece of research work done by **Mr. Hari Shankar Gupta** under our guidance and supervision for the degree of **Doctor of Philosophy in Biotechnology** at Department of Biotechnology, J.C. Bose Institute of Life Science, Bundelkhand University, Jhansi (U.P.) India. That the candidate has put in an attendance of more then 200 days withus.

To the best of our knowledge and belief the thesis:

1. embodies the work of the candidate himself;
2. has been duly completed;
3. fulfills the requirements of the ordinance relating to the Ph.D degree of the University; and
4. is upto the standard both in respect of contents and language for being referred to the examiner.

04.02.2008

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DECLARATION

I, **Hari Shankar Gupta**, hereby declare that the thesis **entitled "Microsatellite enriched genomic library construction, isolation and characterization of microsatellite DNA markers in *Chitala chitala* for population genetics studies"** is my own work conducted under supervision of **Dr. Vinay Singh Chauhan** and Co supervision of **Dr. Peyush Punia** at Department of Biotechnology, J.C. Bose Institute of Life Science, Bundelkhand University, Jhansi (U.P.), approved by Research Degree Committee.

I further declare that to best of my knowledge the thesis does not contain any work, which has been submitted for the award of any degree either in this university or in any other university/deemed university without proper citation.

Date: 04/02/08

Place: Jhansi

(Hari Shankar Gupta)

Dedication

To my
"Mother, Father
and family"

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(Hari Shankar Gupta)

ABBREVIATIONS

AFLP	:	Amplified fragment length polymorphism
AP	:	Alkaline Phosphatase
APS	:	Ammonium per sulphate
ATP	:	Adenosine triphosphate
bp	:	Base pair
CIAP	:	Calf Intestinal Alkaline Phosphatase
cm	:	Centimeter
DDW	:	Double distilled water
DIG	:	Digoxigenin
DNA	:	Deoxyribonuleic acid
dNTP	:	Deoxyribonucleotide triphosphate
E. coli	:	*Escherichia coli*
EDTA	:	Ethylene diamine tetra acetic acid
EST	:	Expressed sequence tag
Fig	:	Figure
g	:	Gram
H_e	:	Expected heterozygosity
H_O	:	Observed heterozygosity
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
KV	:	Kilovolt
MAS	:	Marker assisted selection
mg	:	Miligram
$MgCl_2$	:	Magnesium chloride
ml	:	Mililiter
mM	:	Millimolar
mm	:	milimeter
mtDNA	:	Mitochondrial DNA

n	:	Number of individual
ng	:	Nanogram
P	:	Probability
pBR	:	Plasmid Boliever and Rodriguez
PCR	:	Polymerase chain reaction
pmoles	:	Picomoles
QTL	:	Quantitative trait loci
RAPD	:	Randomly amplified polymorphic DNA
RFLP	:	Restriction fragment length polymorphism
rpm	:	Revolutions per minute
SDS	:	Sodium dodecyl sulphate
SE	:	Standard error
SNP	:	Single nucleotide polymorphism
SSRs	:	Simple sequence repeats
T_a	:	Annealing temperature
TAE	:	Tris acetate EDTA
Taq	:	*Thermophilus aquaticus*
TBE	:	Tris borate EDTA
TE	:	Tris EDTA
TEMED	:	N, N, N,'N'- tetraethyl methylene diamine
T_m	:	Melting temperature
UV	:	Ultraviolet
X Gal	:	5-bromo-4-chloro-3-indolyl- beta-D-galactopyrano
YT	:	Yeast extract tryptone media
µg	:	Microgram
µJ	:	Microjoule
µl	:	Microliter
µM	:	Micromolar

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Introduction

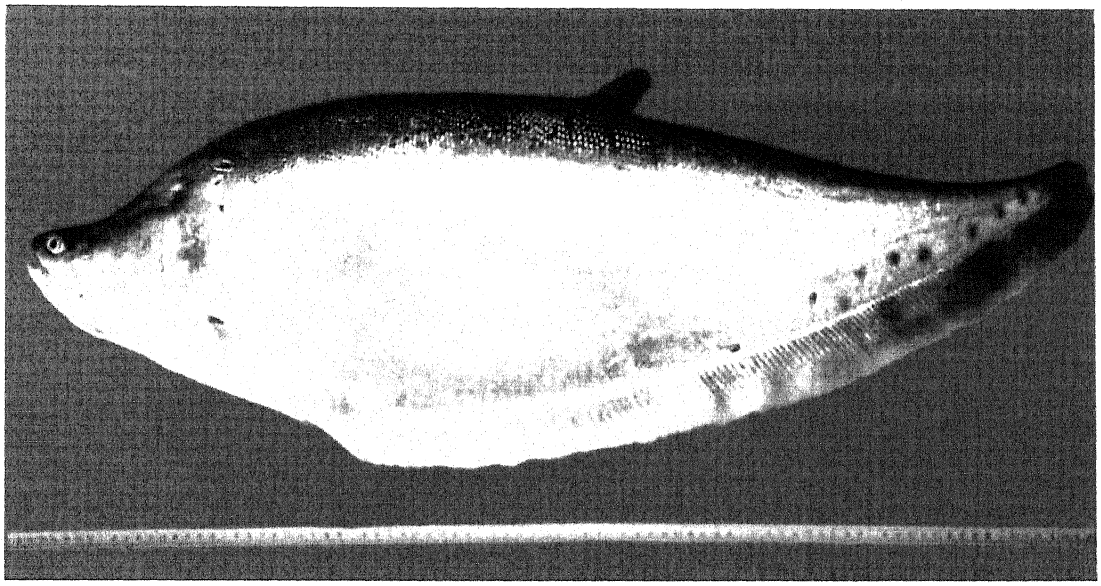
1. INTRODUCTION

Chitala chitala (Photograph. 1) is one of the highly valued fresh water teleostean fish belonging to a primitive order Osteoglossiformes in which some of the fishes are extinct and available in fossil form (Nelson, 1994). The taxonomic hierarchy of *C. chitala* is given as following:

Taxonomic hierarchy of *C. chitala* (Nelson, 1994)

Subphylum Vertebrata (Craniata)
Superclass Gnathostomata
Grade Teleostomi
Class Actinopterygii
Subclass Neopterygii
Division Teleostei
Subdivision Osteoglossomorpha
Order Osteoglossiformes
(6 families, 29 genera and about 217 species)
Suborder Notopteroidei
(4 families, 24 genera and about 209 species)
Superfamily Notopteroidea
Family Notopteridae
(4 genera and 8 species)
Genus *Chitala* (Hamilton, 1822)
(4 species)
Species *Chitala chitala* (Roberts, 1992)

Osteoglossiformes are considered as basal teleosts that preserve primitive anatomical features (teeth on the parasphenoid, tongue bones and numerous other internal characteristics) but their individual member show



Photagragh.1. C*hitala chitala* collected during present study.

peculiar specialization in morphology (elongate anal and dorsal fin), physiology (air breathing function of swim bladder) and behavior (mouth brooding) (Nelson, 1994; Greenwood and Wilson, 1998). Based on amino acid sequences analysis of two mitochondrial protein genes i.e. NADH dehydrogenase subunit 2(ND2) and cytochrome b (cytb) Kumazawa and Nishida (2000) suggested a Paleozoic origin of the order Osteoglossiformes which is considerably older than the first Osteoglossiform fossil record in the Late Jurassic deposits (Benton, 1993). Kumazawa and Nishida (2000) interpreted the apparent discrepancy to be indicative of the paucity of Osteoglossiform fossil records and suspected that there is a long unrecorded history for the Osteoglossiforms in the Mesozoic era. Fossils of bony fishes are not necessarily considered well preserved in general out of 425 teleostean families, 181 (43%) are completely lacking in their fossil record and 58 (24%) of the remaining 244 families having recognizable fossil records that occur only with otoliths (Benton, 1993). Osteoglossomorphes fossil record from Late Jurassic or Early Cretaceous deposits indicates that in the past they were more widely distributed than today (Bonde, 1996; Li and Wilson, 1996a; Li *et. al.*, 1997a, b; Shen, 1996; Taverne, 1998; Zhang, 1998).

Smith *et. al.* (1994) has suggested that all continents remained united as the super continent Pangea during Triassic times (208–245 MYA). In the middle Jurassic (157–178 MYA), Pangea was split into Laurasia and Gondwanaland, which were further fragmented into smaller landmasses of Eurasia, North America, and Greenland from the former, and Africa, South America, Australia, Antarctica, Madagascar, and India from the latter (Smith *et. al.*, 1994; The Plates Project, 1998). Plate tectonics has continuously reshaped these landmasses to the present arrangement. Although extant Osteoglossiformes inhabit terrestrial regions mostly of Godwanian origin, fossil evidences suggests their world wide distribution (Lunderberg, 1993; Bonde, 1996; Li and Wilson, 1996). Freshwater fishes do not disperse easily

through saltwater areas and thus their evolution may be strongly linked to the geological histories of landmasses (Banarescu, 1990; Lundberg, 1993). The Osteoglossomorph fishes are strictly intolerant of saltwater (Banarescu, 1990) and comprise less than 1% of living teleost species (Lavoue and Sullivan, 2004).

Extant Osteoglossiformes are adapted to tropical or sub tropical (Africa, South America, South-East Asia, New Guinea, and North Australia) fresh water habitat with some exception of the North American (family Hiodontidae). The presences of specialized or adaptive features in morphology have contributed to obscure phylogenetic relationships of the Osteoglossiformes (Bonde, 1996; Li and Wilson, 1996). Genetic variation studies through application of molecular markers in the fishes of the order Osteoglossiformes can be of considerable interest as it represents prehistorical lineage. Phylogeny and phylogeography of the Osteoglossomorph is an area of active research. Mitochondrial genome sequences have been used to explore the systematics and phylogenetic relationship in Osteoglossiformes fishes (Kumazawa and Nishida, 2000; Lavoue and Sullivan, 2004). Yue *et. al.* (2004) have studied the genetic diversity of Asian arowana (*Scleropages formosus*) using AFLP and microsatellites.

Nelson (1994) divided Osteoglossiformes into two suborders, i.e., Osteoglossoidei and Notopteroidei. The former comprises arowanas (the family Osteoglossidae) and the butterfly fish (Pantodontidae), whereas the letter includes mooneyes (Hiodontidae), Old world knifefishes (Notopteridae), elephantfishes (Mormyridae), and the aba (Gymnarchidae). The family Notopteridae contains four genera and eight species of Osteoglossiform fishes, commonly known as featherbacks and knifefishes (Roberts, 1992). Genera *Xenomystus* (one species, *X. nigri*) and *Papyrocranus* (two species, *P. afer* and *P. congoensis*) are African, whereas the genera *Notopterus* (one species, *N. notopterus*) and *Chitala* (four species, *C. chitala*, *C. blanci*, *C. lopis*, and *C.*

ornata) occur in South and Southeast Asia. Members of family Notopteridae are elongated in shape and their fins run the length of the body, with the resulting appearance of a knife. Most previous studies addressing the phylogenetic systematics of this family support the monophyly of the Notopteridae, but the incomplete fossil record of Notopterids provides few insights into the relationships among these living taxa. The recent discovery and description of *Palaeonotopterus greenwoodi* (Forey, 1997; Taverne, 1998; Taverne and Maisey, 1999) from the Upper Albian or Lower Cenomanian (100 million years before present) of Morocco indicates a more ancient origin of this group than previously imagined (Rana, 1988; Bonde, 1996; Li and Wilson, 1996b). The ambiguity regarding the systematics of family Notopteridae has been reviewed several times (Lim and Furtado, 1986; Roberts, 1992; Guo-Qing *et. at.*, 1997). Roberts (1992) placed *C. chitala*, *C. ornata*, *C. blanci* and *C. lopis* under new genus *Chitala* while *N. notopterus* was retained with original genus *Notopterus.* Recent studies by RAPD and allozyme markers (Lal *et. al.*, 2006) confirm that genus *Chitala* is different from *Notopterus*. Sodsuk and Sodsuk (2000) studied allozyme variation in feather-back fish in Thailand and found *C. ornata*, *C. blanci* and *C. lopis* genetically close to each other than *N. notopterus*.

Chitala chitala which commonly known as Indian feather-back and previously as *Notopterus chitala* (Hamilton, 1822), is widely distributed in freshwater bodies of Indian subcontinent including Bangladesh, Myanmar, Nepal, and Pakistan (Froese and Pauly, 2003). Indian natural ranges of distribution include Ganga, Bhramaputra, Mahanadi and Narmada river systems, as well as other east and west coastal rivers are also more or less populated with this species. Basically *C. chitala* is a rheophilic fish, but it has established itself in artificial lakes and confined waters while the natural habitat of *C. chitala* includes large rivers, streams, reservoirs, lakes, ponds, etc. *C. chitala* is easily distinguishable from in body coloration and shape.

Morphologically these are oblong and laterally compressed with terminal mouth and prominent snout. The humpback margins along the dorsal ridge and the black spots near the tail are the characteristics features of *C. chitala*. These spots and markings are not clear or absent in some locations, which may be due to varying environmental conditions in different habitats. Small cycloid scales are present which are larger on head than those on body whereas the tail is prolonged and tapering. *C. chitala* is reported to grow upto 150 cm and breed in the season of late May to July. *C. chitala* is known for its nutritive value and delicious flesh quality particularly in the eastern region of India thus commands a high market value as food fish (Lilabati and Vishwanath, 1998) as well as for ornamental trade (Hardwick, 2005). Due to economical value *C. chitala* has undergone heavy fishing pressure, leading to an alarming decline in the natural populations and is at present has been categorized as endangered, following IUCN criteria (CAMP, 1997; Goswami, 2000; *Sarkar et. al.*, 2006). Nominal world wide catches of feather-back fish species were 5,173 mt in 1995, where as in 1998 it came down to 3,194 mt (FAO, 2000) thus indicating a need of conservation and management for the species in its natural habitats. Therefore, research on artificial propagation for developing culture and *in situ* conservation is being pursued actively (Sarkar *et. al.*, 2006). Data on genetic variations can provide crucial input to plan the effective strategies for conservation and rehabilitation of natural populations. The conservation of genetic variation is an essential component of many species management programmes and to manage any biological resources effectively, researcher must identify the level of genetic variation within and among populations.

Genetic variability is directly assessed through molecular markers and technological advances in molecular biology and biochemistry have led to the development of a variety of genetic markers that can be used to address the questions of relevance to the management and conservation of fish species (Ferguson and Danzmann, 1998). Genetic markers have been applied to the

fisheries particularly in stock structure analysis, aquaculture, and taxonomic/systematics (Ward and Grewe, 1994) with varying degree of success (Carvalho and Hauser, 1994). Genetic markers can be categorized based on their transmission and evolutionary dynamics (Park and Moran, 1994). Markers such as allozymes, randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), minisatellite and microsatellite loci are biparentlly inherited, whereas mitochondrial DNA (mtDNA) are non recombining and maternally inherited.

Genetic research on *C. chitala* is limited so far and karyotypic studies identified 12 metacentric and 36 acrocentric chromosomes. Total number of diploid chromosome is 48 (Nayyar, 1966). Literature survey indicates the lack of information of genetic variability and population structure information of *C. chitala*. The molecular markers suitable to detect genetic variation in this primitive fish species are also not known. Mandal (2005) highlights the possible utility of microsatellite markers for genetic divergence studies of C. *chitala* because allozyme analysis did not reveal any polymorphic locus and two microsatellite loci isolated through cross species amplification found polymorphic but were not sufficient to study genetic variation. Microsatellite loci consist of simple tandemly repeated sequence of 1 to 6 bp in length (Litt and Luty, 1989; Tautz, 1889; Weber and May, 1989). Owing to the variation in number of repeat units, microsatellites may exhibit high degree of length polymorphism. Microsatellite primers from one species can be used for amplification of polymorphic microsatellite loci from species of same family or cross species. But sometimes the primers which give polymorphic loci for one species do not generate polymorphic loci in other species. This becomes a major hindrance to study genetic variation and hence require development of suitable microsatellite markers for study of genetic variations (Rubinsztein *et. al.*, 1995; Morin *et. al.*, 1998). Microsatellites enriched libraries have been constructed for many organisms, including fishes, *Tor tambroides* (Nguyen *et.*

al., 2007), *Sepia esculenta* (Zheng *et. al.*, 2007), *Scarus rubroviolaceus* (Carlon and Lippe, 2007) etc.

The present study is being undertaken with the objective of constructing microsatellite enriched genomic library of *C. chitala* to isolate and characterize microsatellite markers and to study distribution as well as the pattern of genetic variation in *C. chitala*

The main objectives of the study are:

(a) Construction of Microsatellite enriched genomic library of *Chitala chitala.*

(b) Isolation of microsatellite repeats sequences in the genome of *Chitala chitala* using genomic library.

(c) Characterization and primer designing of identified microsatellites.

(d) Genetic variability analysis between and within the natural populations using the identified polymorphic microsatellite loci.

(e) Population structure analysis across the distribution range of *Chitala chitala* in India.

Review of literature

2. REVIEW OF LITERATURE

Biodiversity or biological diversity is the diversity of life which refers to variation at all level of biological organization (Kevin and Spicer, 2004). Diversity includes diversity within species, among species and comparative diversity among ecosystems. The foundation for biodiversity and organic evolution is the genetic variation within species. Genetic variation is an important feature of populations both for short term fitness of individual and the long term survival of the population by allowing adaptation to changing environment conditions to occur. Genetic variation is similarly important in farmed populations allowing selective breeding and preventive loss of fitness due to inbreeding depression. In small isolated populations genetic variability can be substantially reduced through genetic drift and inbreeding and such reduction may result in the decrease fitness and eventual extinction. If the deleterious effects of breeding are to be avoided, crossing fish from genetically different strains is of critical importance. This can be done effectively if knowledge about genetic similarity or difference between strains is available, especially when pedigree information is lacking (Ferguson, 1994). In conjunction with other evolutionary forces like selection and genetic drift, genetic variation arises between individuals leading to differentiation at the level of population, species and higher order taxonomic groups. Molecular genetic markers are powerful tools to detect genetic uniqueness of individuals, populations or species (Avise, 1994; Linda and Paul, 1995). These markers have revolutionized the analytical power necessary to explore the genetic diversity (Hillis *et. al.*, 1996). The conclusion from genetic diversity data has varied application in research on evolution, conservation and management of natural resources, genetic improvement programmes etc (Ferguson *et. al.*, 1995; Neff and Gross, 2001; Jehle and Arntzen, 2002; Wasko *et. al.*, 2003; Morin *et. al.*, 2004; Liu and Cordes, 2004).

Molecular markers can be classified into type I and type II markers. Type I are markers associated with genes of known function, while type II markers are associated with anonymous genomic segments (O'Brien, 1991). Microsatellite markers are type II markers unless they are associated with genes of known function. Type I markers have utility in studies of comparative genomics, genome evolution and candidate gene identification. Type I markers serve as a bridge for comparison and transfer of genomic information from a map rich species into a relatively map-poor species. In general, type II markers such as RAPDs, microsatellites, and AFLPs are considered to be non-coding and therefore selectively neutral. Such markers have found widespread use in population genetic studies whose characterizations of genetic diversity and divergence within and among populations are based on assumptions of Hardy–Weinberg equilibrium and selective neutrality of the markers employed (Brown and Epifanio, 2003). In case of microsatellite markers, such comparative studies depend on conservation of the flanking sequences used for the design of PCR primers. Type II markers also have proven useful in aquaculture genetics for species, strain and hybrid identification, in breeding studies, and more recently as markers linked to QTL. Such markers have found widespread use in population genetic studies to characterize genetic divergence within and among the populations or species (Brown and Epifanio, 2003).

The potential of molecular markers to fisheries management has long been recognized (Utter, 1991). The early appreciation of the value of genetic markers is in a large part due to the challenges of observing behavior and migration patterns in an aquatic/marine environment. Early studies on the molecular phenotypes used blood group polymorphisms to discriminate between spatially discrete populations of fish (Sick, 1961). Because of the problem of interpretation, research turned to specific histochemical stain procedures (Hunter and Markert, 1957). Staining for specific proteins was

used in association with starch gel electrophoresis and permitted the detection of allozyme variation (Harris and Hopkinson, 1976). In the late 1970s workers start investigating of DNA sequences and first focused on mitochondrial DNA (mtDNA) molecule. However more recent marker types that are finding service in this field include, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers.

2.1 Types of molecular markers

2.1.1 Allozyme markers

Allozymes are allelic variants protein product by single gene locus and are of interest as marker because existing polymorphism and recent protein products of genes and thus type I markers. Since the 1960s, the starch gel electrophoresis of allozymes has been the most commonly employed molecular method in fishery genetics using protein-coding loci (Ryman and Utter, 1987; Hillis *et. al.*, 1996). Allozyme were among the earliest markers used in aquaculture genetics (May *et. al.,* 1980; Seeb and Seeb, 1986; Johnson *et. al.,* 1987; Liu *et. al.*, 1992, 1996; Morizot *et. al.*, 1994) and still in wide spread use. Amino acid differences in the polypeptide chains of the different allelic forms of an enzyme reflect changes in the underlying DNA sequences. Depending upon the nature of the amino acid changes the resulting protein product may migrate at different rates and differences in the presences/absence and the relative frequency of alleles are used to quantify genetic variations and distinguish among genetic units and the levels of population species and higher taxonomist designation (Liu and Cordes, 2004). Allozyme found use in aquaculture for tracking inbreeding, stock identification, and parentage analysis. In a few cases, correlations existed between certain allozyme markers and performance traits (Hallerman *et. al.*, 1986; McGoldrick and Hedgecock,

1997). Their use in linkage mapping has been demonstrated in studies of salmoids (Pasdar *et. al.,* 1984; May and Johnnson, 1993) and poeciliids (Morizot *et. al.,* 1991). Murphy *et. al.* (1990) list 75 enzymes systems coded by several genetics loci that may potentially be analyzed in fishes. Disadvantage associated with the allozymes include null (enzymetically inactive) alleles, amount and freshness of tissue sample required. Some DNA sequence changes are masked at the protein level, reducing the level of detectable variation and low level of genetic variation revealed in many allozyme studies of marine fish population (Siddell *et. al.,* 1980; Mork *et. al.,* 1985; Crawford *et. al.,* 1989) so in spite of their strength as codominant Type I markers, ease of use and low cost their use in aquaculture has become limited.

2.1.2 Mitochondrial DNA (mtDNA) markers

By the early 1980's examination of the gene itself became possible by determining directly or indirectly difference in the nucleotide sequence of DNA molecule. One of the finding that arose from early studies was that the DNA of mitochondria (mtDNA) is characterized by high level of sequencing diversity at the species or infra-species level despite great conservation of gene function and arrangement (Wirgin and Waldam, 1994). Mitochondrial DNA became a very popular marker and dominated genetic studies designed to answer questions of phylogeny and population structure in fish for more than a decade. Three properties of mtDNA set apart from nuclear DNA: it occurs in multiple copies in each cell (in contrast to two copies for a "single copy" nuclear locus), it is transmitted uniparentally, and it does not recombine. Moreover it evolves, much faster than coding regions of DNA (Brown *et. al.,* 1982; Attardi, 1985; Moritz *et. al.,* 1987; Avise, 1994). Presence of multiple copies does not however translate into a large variety of copies within the cell. For reasons not fully understood the speed with which the maternal lineage of a heteroplasmically conceived individual becomes homoplasmic is rather high.

As a result, we can speak of the "mitotype" of an individual in the same way as we speak of its (nuclear) genotype. One consequence of uniparental transmission is that the effective population size for mtDNA is smaller than that of nuclear DNA (Moritz *et. al.,* 1987), so that mtDNA variation is a more sensitive indicator of population phenomena such as bottlenecks and hybridizations. Sex-specific differences in gene flow could also be revealed by contrasting nuclear with mitochondrial DNA. In a species in which mtDNA is maternally transmitted but gene flow occurs mainly or exclusively through males, divergence among populations is expected to be much higher for mtDNA than nuclear DNA. This at the same time means that a disadvantage of this marker in its inability to detect male mediated genetic mixing of stocks. Analyses of mtDNA markers have been used extensively to investigate stock structure in a variety of vertebrates including fishes (Avise *et. al.*, 1986; Graves *et. al.*, 1992; Gold *et. al.*, 1993; Chow *et. al.*, 1993; Heist and Gold, 1999), birds (Baker and Marshall, 1997; Greenberg *et. al.*, 1998; Mila *et. al.*, 2000; Zink *et. al.*, 2000), mammals (Menotti-Raymond and O'Brien, 1993) and reptiles (Avise *et. al.*, 1998; Serb *et. al.*, 2001; Riberon *et. al.*, 2002; Shanker *et. al.*, 2004).

2.1.3 Random amplified polymorphic DNA (RAPD) markers

RAPD procedures were first developed in 1990 using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 8-10 bp in length (Welsh and McClelland, 1990; Williams *et. al.,* 1990). Because the primers are short and relatively low annealing temperatures (often 36-40°C) are used, the likelihood of amplifying multiple products is great, with each product (presumably) representing a different locus. Genetic variation and divergence within and between the taxa of interest are assessed by the presence or absence of each product, which is dictated by changes in the DNA sequence at each locus. RAPD polymorphisms can occur due to base substitutions at the

primer binding sites or to indels in the regions between the sites. The potential power is relatively high for detection of polymorphism; typically, 5-20 bands can be produced using a given primer pair and multiple sets of random primers can be used to scan the entire genome for differential RAPD bands (Liu and Cordes, 2004).

Shortcomings of this type of marker include the difficulty of demonstrating Mendelian inheritance of the loci and the inability to distinguish between homozygotes and heterozygotes. Analysis follows the assumption that populations under study follow Hardy-Weinberg expectations. In addition, the presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus), low reproducibility due to the low annealing temperature used in the PCR amplification, have limited the application of this marker in fisheries science (Wirgin and Waldman, 1994).

2.1.4 Amplified fragment length polymorphism (AFLP)

AFLP is a PCR-based, multi-locus fingerprinting technique that combines the strengths and overcomes the weaknesses of the RFLP and RAPD methods. Like RFLPs, the molecular basis of AFLP polymorphisms includes indels between restriction sites and base substitutions at restriction sites; like RAPDs, it also includes base substitutions at PCR primer binding sites. The unique feature of the technique is the addition of adaptors of known sequence to DNA fragments generated by digestion of whole genomic DNA. This allows for the subsequent PCR amplification of a subset of the total fragments for ease of separation by gel electrophoresis. Its primary target of genetic variation is the same as RFLP, but instead of analyzing one locus at a time, it allows for the analysis of many loci simultaneously.

First employed by Vos *et. al.* (1995) AFLP generation begins with the digestion of whole genomic DNA with two enzymes (most often *Eco*RI and *Mse*I). Since sequences for the resulting DNA fragments are unknown,

adaptors of known sequence are ligated to the ends of the fragments and used as prime sites for PCR amplification. As this would result in the production of millions of PCR fragments, the number of amplified fragments is reduced by adding known bases to the 3' of the PCR primers. The power of AFLP analysis is tremendously high for revealing genomic polymorphisms. For instance, Young *et. al.* (2001) used the AFLP technique generate 133 polymorphic markers, 23 of which were diagnostic in distinguish rainbow trout, coastal cutthroat trout, and their hybrids. AFLP markers also have used for analysis of meiogynogens and androgens (Young *et. al.,* 1996; Felip *et. al.,* 2000).

2.1.5 Single nucleotide polymorphism (SNP) markers

Single nucleotide polymorphism (SNP) describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. Such sequence differences due to base substitutions have been well characterized since the beginning of DNA sequencing in 1977, but the ability to genotype SNPs rapidly in large numbers of samples was not possible until the application of gene chip technology in the late 1990s. SNPs are again becoming a focal point in molecular marker development since they are the most abundant polymorphism in any organism, adaptable to automation, and reveal hidden polymorphism not detected with other markers and methods (Morin *et. al.*, 2004; Liu and Cordes, 2004). Despite technological advances, SNP genotyping is still a challenging endeavor and requires specialized equipment. Traditional methods available for SNP genotyping include: direct sequencing, single base sequencing (Cotton, 1993), allele specific oligonucleotide (Malmgren *et. al.,* 1996), denaturing gradient gel electrophoresis (Cariello *et. al.,* 1988), single strand conformational polymorphism assays (Suzuki *et. al.,* 1990), and ligation chain reaction (Kalin *et. al.,* 1992). Each approach has its advantages and

limitations. More detailed information concerning SNP genotyping available in a recent review by Vignal *et. al.* (2002).

2.1.6 Expressed sequence tags (ESTs) markers

Expressed sequence tags (ESTs) are single-pass sequences generated from random sequencing of cDNA clones (Adams *et. al.*, 1991). The EST approach is an efficient way to identify genes and analyze their expression by means of expression profiling (Franco *et. al.*, 1995; Azam *et. al.*, 1996; Lee *et. al.*, 2000). It offers a rapid and valuable first look at genes expressed in specific tissue, types, under specific physiological conditions or during specific developmental stages. ESTs are useful for the development of cDNA microarray that allow analysis of differentially expressed genes to be determined in a systematic way (Schena *et. al.*, 1996; Wang *et. al.*, 1999), in addition to their great value in genome mapping (Boguski and Schuler, 1995; Hudson *et. al.*, 1995; Schuler *et. al.*, 1996). For genome mapping, ESTs are most useful for linkage mapping and physical mapping in animal genomics such as those of cattle and swine, were radiation hybrid panels are available for mapping non polymorphic DNA marker (Cox *et. al.*, 1990). EST sequences are archived in a special branch of the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/dbEST/index.htrnl) (Wheeler *et. al.*, 2004). In November 2005, the EST database contained more than 31.3 million sequence entries from around 500 species. Much progress has been made in fisheries for analysis of ESTs. Tissue analysis of ESTs and expression profiling has been conducted in channel catfish (Karsi *et. al.*, 1998, 2002a; Ju *et. al.*, 2000; Cao *et. al.*, 2001; Kocabas *et. al.*, 2002). Recently, major progress has been made toward EST development in several aquaculture species, especially in Atlantic salmon and rainbow trout, where over 100,000 ESTs have been sequenced (Davey *et. al.*, 2001; Martin *et. al.*, 2002; Rexroad, 2003; Rexroad *et. al.*, 2003; Rise *et. al.*, 2004).

2.1.7 Tandemly Repeated DNA

Tandemly repeated blocks of DNA of identical or similar sequence are dispersed through out the genome of most if not all, eukaryotic genome (O'Reilly and Wright, 1995). Three different class of this repetitive and highly polymorphic DNA have been distinguished traditionally, based on the size of the repeat unit.

2.1.7.1 Major Satellite arrays

Satellite DNA in which a single repeat sequence family can constitute several percent of the total genome, and can occur in individual repeat area even of size 5Mb. Satellites (and in particular the alphoid family) are often preferentially associated with centromeres. Major satellites are only infrequently used to genotype individuals, but have been useful in human genome mapping in providing genetic markers anchored at centromeres. They can be typed either by Southern blot/hybridization (Mahtani and Willard, 1990; Oakey and Tyler-Smith, 1990), or using restriction digests or PCR primers which detect locus specific repeat unit variants (Warburton and Willard, 1996).

2.17.2 Minisatellite markers

Minisatellite or variable Number of tandem repeat (VNTR) DNA (Warburton and Willard, 1996) which may be present at hundreds or thousands of different loci per genome in which a repeat unit sequence long enough (> 10 bp) to be locus specific is repeated to give repeat blocks of intermediate size (0.5-30 kb). The term "DNA fingerprinting" was originally associated with the approach of Jeffreys *et. al.* (1985), in which Southern blot/hybridization assays of minisatellite regions of DNA (after restriction digestion of individual genomic DNA) reveal multilocus gel banding profiles that distinguish most or all individuals within a sexually reproducing species (Avise, 1994). The original Jeffrey's probes, which hybridized to conserved core sequences, 10-15

bp long, were isolated from a myoglobin intron in humans, but it was found that they also cross-hybridized in many other species, including fishes (e.g., Baker *et. al.,* 1992). Subsequently there has been an explosion in the development of new methods for the production of species and individual specific DNA fingerprints. However, profiles resulting from multi-locus DNA fingerprinting are very complex and this puts severe constrains in the analysis and interpretation of the results. It is almost impossible to identify both members of allelic pairs at individual loci and therefore estimation of allelic frequencies cannot be obtained. These limitations render multilocus fingerprinting rather unsuited for population level applications (Wright, 1993). Moreover, quite often the results of multi-locus fingerprinting protocols are not reproducible. As a result, the development of single-locus profiling techniques was sought, in which allelic variation is surveyed at individual VNTR loci. Two approaches have been used: a) Southern blotting and hybridization using as a probe DNA from a single VNTR locus, preferably the unique flanking regions, and b) by PCR amplification of the locus using primers flanking the repeat array and separation of the PCR products by gel electrophoresis (O'Reilly and Wright, 1995). Single-locus approaches obviate most of the problems associated with multi-locus methods; nevertheless some limitations still exist, mainly because often alleles do not differ from one another by discrete, integral increases or decreases in the number of repeat copies (Jeffreys *et. al.*, 1988). This makes the comparison of alleles sizes between gels difficult, and has necessitated the binning of alleles into defined size classes (O'Reilly and Wright, 1995).

2.1.7.3 Microsatellite markers

Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (e.g., ACA or GATA; Tautz, 1989; Litt and Luty, 1989). Abundant in all species studied to

date, microsatellites have been estimated to occur as often as once every 10 kb in fishes (Wright, 1993). Microsatellites tend to be evenly distributed in the genome on all chromosomes and all regions of the chromosome. They have been found inside gene coding regions (Liu *et. al.*, 2001c), introns, and in the non-gene sequence. The best known examples of microsatellites within coding regions are those causing genetic diseases in humans, such as the CAG repeats that encode polyglutamine tract, resulting in mental retardation. Most microsatellite loci are relatively small, ranging from a few to a few hundred repeats. Database analyses of tandem repeats in genomic sequences by Beckmann and Weber (1992) showed that CA/TG repeats are the most common dinucleotide repeats, occurring about twice as frequently as AT repeats and three times as often as AG/TC repeats. Microsatellite polymorphism is based on size differences due to varying numbers of repeat units contained by alleles at a given locus. Microsatellite mutation rates have been reported as high as 10^{-2} per generation (Weber and Wong, 1993; Crawford and Cuthbertson, 1996), and are believed to be caused by polymerase slippage during DNA replication, resulting in differences in the number of repeat units (Levinson and Gutman, 1987; Tautz, 1989). Direct studies of human families have shown that new microsatellite mutations usually differed from the parental allele by only one or two repeats (Weber and Wong, 1993), favoring a stepwise mutation model (Estoup and Cornuet, 1999). However, in a few fish species alleles with very large differences in repeat numbers have been observed, predictive of an infinite allele model (Balloux and Lugon Moulin, 2002)

Microsatellites are inherited in a Mendelian fashion as codominant marker, which is strength of microsatellite markers in addition to their abundance, even genomic distribution, small locus size, and high polymorphism. However, use of microsatellite markers involves a large amount of upfront investment and effort. Each microsatellite locus has to be

identified and its flanking region sequenced for the design of PCR primers. Because of PCR based technique significant increases in the number of samples that can be typed in a day have been achieved by using automated fluorescent sequencers coupled with computer imaging systems (O'Reilly and Wright, 1995).

Evolutionary analyses of microsatellites have shown a wide variety of degrees of conservation. Microsatellite primers from one species can be used for amplification of polymorphic microsatellite loci from species of same family or cross species. Studies have shown that cross species amplification using primers designed from related species generate polymorphic loci to identify different populations and their genetic viabilities (Welsh *et. al.*, 1990; Williams *et. al.*, 1990). The presence of highly conserved flanking regions has been reported for some microsatellite loci in cetaceans (Schlotlerer *et. al.*, 1991), turtles (FitzSimmons *et. al.*, 1995) and fish (Rico *et. al.*, 1996), allowing cross-amplification from species that diverged as long as 470 million years ago. It should be noted that during the isolation procedure, loci are selected from the upper end of the repeat length distribution in the genome, the fraction which is known to harbor the most polymorphic markers (Primmer *et. al.*, 1996). Such bias in loci isolation may likely result in a lower level of polymorphism when orthologous loci are tested in other species (Ellegren *et. al.*, 1995). Therefore, high polymorphism observed in a species does not guarantee that similar polymorphism will be found in related species especially when increasing the evolutionary distance and this becomes a major hindrance to study genetic variation hence required isolation of microsatellite loci development of suitable primers for study of variations (Rubinsztein *et. al.*, 1995; Morin *et. al.*, 1998).

Microsatellites enriched libraries have been constructed for many organisms, including fishes. The high frequency of tandem repeats in fish genomes provides a good opportunity to obtain libraries significantly enriched

in microsatellites. For example, libraries containing 74%, 95% and 96% clones with $(CA)_n$ repeats have been developed for the Mediterranean angler fish *Lophius* sp. (Garoia *et. al.*, 2003), gilthead sea bream *Sparus aurata* (Zane *et. al.*, 2002), and *Nile tilapia* (Carleton *et. al*, 2002), respectively. A library usually contains 1000-4000 recombinant clones. Screening of these clones typically yields 10-15% unique polymorphic SSRs, resulting in the production of 100-500 non-redundant variable microsatellites from a single library (Zane *et. al.*, 2002). The recent debut of the journal Molecular Ecology Notes, dedicated almost entirely too publishes primer and allele frequency data for newly characterized microsatellite loci in a wide range of species.

2.2 Available methods for microsatellite isolation

A number of new protocols have appeared in the literature in the last few years. These methods often present only slight differences from one another and used frequently by different workers.

2.2.1 Traditional Method

Microsatellite loci have been isolated starting from a partial genomic library of the target species (Rassmann *et. al.*, 1991). High quality genomic DNA is fragmented either using restriction enzymes or less commonly by sonication. In the former case, the choice of the restriction enzyme depends on the desired average length of DNA fragments, the microsatellite repeat to be found, and the type of ends (cohesive or blunt) of the restriction fragments. Fragmented DNA is then size-selected to preferentially obtain small fragments (300-700 bp). Depending on the fragmentation method, DNA fragments are ligated into a common plasmid vector either directly or after ligation to specific adaptors. Transformation of bacterial cells with ligation product generally yields thousands of recombinant clones that can be subsequently screened for the presence of microsatellite sequences. The

numbers of positive clones (containing microsatellite) that can be obtained by this traditional method usually range from 0.04% to 12%. A different approach (PCR isolation of microsatellite arrays; PIMA), which skips all steps from DNA fragmentation to cloning, has been proposed by Lunt *et. al.* (1999). Briefly, several RAPD primers are used to obtain randomly amplified fragments from the target species genome. These amplicons are then cloned by using a TA vector and arrayed clones are screened using repeat specific and vector primers. This and similar techniques (Ender *et. al.*, 1996; D' Amato *et. al.*, 1999) take advantage of the fact that RAPD fragments seem to contain microsatellite repeats more frequently than random genomic clones (Cifarelli *et. al.*, 1995).

2.2.2 Primer extension reaction

Protocol has been developed that produce genomic libraries that are highly enriched for specific microsatellite repeats using a primer extension reaction (Ostrander *et. al.*, 1992; Paetkau 1999). Method relies on the construction of a primary genomic library, in which fragmented genomic DNA are inserted into a phagemid or a phage vector in order to obtain a single strand DNA (ssDNA) library. ssDNA is then used as a template for a primer extension reaction, primed with repeat-specific oligonucleotides, which generates a double strand product only from vectors containing the desired repeat. Transformation of bacterial cells with ligation product generally yields thousands of recombinant clones that can be subsequently screened for the presence of microsatellite sequences. The two enrichment procedures diverge in the strategy used to recover primer-extended products. In the Ostrander and coworkers approach, 40,000–60,000 colonies from a phagemid library are eluted from the primary library and after the selective conversion of ssDNA to double strand DNA through $(CA)_n$ or $(GT)_n$ primer extension, the mixture is used to transform a *Escherichia coli* strain. With 60,000 clones in the primary

library, in the case of a specific repeat motif with genomic frequency lower than 1%, only 600 loci (containing the desired repeat motif) will be represented in the enriched library.

In the Paetkau protocol the primary library is obtained using M13 phage, and circular ssDNA is obtained through elution of 30,000 clear plaques. Primer extension is then performed using 5' biotinylated oligonucleotides and Klenow DNA polymerase. This reaction results, for microsatellite containing phages, in a population of circular DNA molecules whose second strand is a linear primer-extended molecule of DNA with a biotin at one end. These products are selectively recovered from the reaction mix using streptavidin coated beads and after washing steps, circular phage ssDNA is released by denaturation. Finally, molecules containing the microsatellites are converted to double-stranded molecules with a second round of primer extension and are then used for the final transformation same as in Ostrander method.

2.2.3 Selective hybridization

Selective hybridization protocols appear to be extremely popular as it is being used in over 25% of all reviewed primer notes and 70% of those employing enrichment procedures (Zane *et. al.*, 2002). The basic protocol as proposed by Karagyozov *et. al.* (1993), Armour *et. al.* (1994), Kijas *et. al.* (1994) is relatively straightforward, although several modifications have been independently suggested by various authors in an attempt to further optimize crucial steps or to remove unnecessary procedures. A very simple strategy for microsatellite isolation using selective hybridization can be outlined based on several reports that have been published (Karagyozov *et. al.*, 1993; Hamilton *et. al.*, 1999). The first step is identical to traditional isolation procedures, aimed at producing small genomic fragments that are then ligated to a known sequence, a vector or an adaptor. Following the fragmentation-ligation step,

and depending on the amount of starting DNA, the DNA is hybridized (if necessary after amplification) with repeat containing probe. The probe can be bound to a nylon membrane (Karagyozov *et. al.*, 1993; Armour *et. al.*, 1994) or 5' biotinylated and bound to streptavidin coated beads (Kandpal *et. al.*, 1994; Kijas *et. al.*, 1994). After the hybridization step and several washes to remove nonspecific binding, the DNA is eluted and recovered by PCR amplification. Finally, the enriched DNA cloned into a suitable vector, either by using a restriction site on the known flanking regions or by TA, cloning. Depending on the efficiency of the whole procedure, recombinant clones can be directly sequenced or screened for the presence of repeats by using colony hybridization or PCR strategies.

2.2.4 FIASCO (Fast isolation by AFLP of Sequences containing repeats)

Zane *et. al.* (2002) tested this procedure in different organisms such as birds (*Passera lagia*), fish (*Sparus aurata* and *Lophiusamericanus*), crustacean (*Meganyctiphanes norvegica*) and red coral (*Corallium rubrum*). The percentage of clones containing dinucleotide repeats varied from a minimum of 50% *Passera lagia*) to a maximum of 95% (*Sparus aurata*). The method is fast and simple, and many unnecessary steps have been eliminated. The protocol relies on the extremely efficient digestion-ligation reaction of the amplified fragment length polymorphism procedure. DNA is simultaneously digested with *Mse*I and ligated to *Mse*I AFLP adaptor. Amplification is performed by using a primer with a selective nucleotide at the 3' end that matches the first nucleotide beyond the original restriction site. Amplified molecules hybridized to biotinylated probes are selectively captured by streptavidin coated beads. Amplification and cloning of hybridized inserts can be conveniently carried out by using the TOPO-TA cloning kit with an expected yield of 1000–4000 recombinant colonies.

With regard to isolation protocols, while most authors remain faithful to the basic methods of library screening, a substantial fraction of papers (Zane *et. al.*, 2002) describe the use of enhanced protocols. Such use seems to be biased with respect to the investigated species. For some taxonomic groups, microsatellite-enriched libraries are commonly employed, whereas in other taxa they are less frequently used. Details of available strategies for microsatellite isolation have been reviewed by Zane *et. al.* (2002).

2.2.5 EST based Type I microsatellite markers

The most effective and rapid way for producing type I micro satellites is the sequencing of clones from cDNA libraries. Both 5' and 3'ends of a cDNA clone can be sequenced to produce expressed sequence tags (ESTs). An EST represents a short, usually 200-600 bp-long nucleotide sequence, which represents a uniquely expressed region of the genome. SSRs can be searched for in these EST sequence databases. However, the major drawback for effective and rapid development of type I SSRs is access to sufficient sequence information. In channel catfish, around 45,000 EST sequences have been developed. This provides a serious source for extracting thousands of sequences containing putative SSRs with the possibility of developing several hundreds of polymorphic microsatellite markers. For example, sequence analysis of 1909 ESTs from a skin cDNA library of *Ictalurus punctatus* revealed the presence of 89 (4. 7% of 1909) microsatellite containing genes (Karsi *et. al.*, 2002). A recent bioinformatic analysis of 43,033 ESTs from channel catfish revealed 4855 ESTs (11.3%) containing microsatellites (Serapion *et. al.*, 2004). The dinucleotide CA/TG and GA/TC pairs were the most abundant among EST-derived microsatellites (Serapion *et. al.*, 2004).

A typical strategy for the development of EST derived microsatellite markers (data mining) includes preliminary analysis of EST sequences from

the DNA database to remove poly(A) and poly(T) stretches. These mononucleotide repeats are very common in ESTs developed from the 3'-ends of cDNA clones and correspond to the poly(A) tails in eukaryotic mRNA. Sequences are further screened for putative SSRs. Following the identification of micro satellite-containing ESTs, flanking primers should be designed to amplify a microsatellite. In order to hypothesize about putative functions of SSR containing genes, these sequences are needed for comparison to the database of amino acid sequences (Kantety *et. al.*, 2002; Thiel *et. al.*, 2003). UniProt/Swiss-Prot is an annotated protein sequence database (http://www.ebi.ac.uk/swissprotl) which is extremely helpful for these purposes.

2.3 Applications of microsatellites markers

Because of multiallelic nature, codominant inheritance, small length, extensive genome coverage and relative abundance, microsatellites have been successfully applied in a wide variety of research fields and practical disciplines (Powel *et. al.*, 1996).

2.3.1 Genetic mapping

Linkage maps are known as recombination maps and define the order and distance of loci along a chromosome on the basis of inheritance in families or mapping populations. SSRs remain the markers of choice for the construction of linkage maps, because they are highly polymorphic and require a small amount of DNA for each test. A disadvantage of microsatellites is that they are mostly anonymous DNA fragments (Cullis, 2002). However, type II (noncoding) microsatellites are very helpful for building a dense linkage map framework into which type I (coding) markers can then be incorporated. Compared to type II markers, mapping type I markers directly shows the location of genes within the linkage map. Therefore, enrichment of the linkage

map by type I loci greatly benefits the mapping and characterization of genes responsible for medically, agriculturally and evolutionarily important complex traits. This also provides a good opportunity for marker assisted selection (MAS) in commercially significant species (Poompuang and Hallerman, 1997; Waldbieser and Wolters, 1999). During linkage map construction, co-segregating markers are placed into linkage groups, and the proportion of recombinants detected between linked markers is used as a measure of distance between them. Genetic distance is usually measured in centimorgans (cM), where 1 cM is equivalent to 1% recombination between markers. Consolidated linkage maps have been published for fishes, such as Arctic chaff *Salvenius alpinus* (Woram *et. al.*, 2004), Atlantic salmon (Moen *et. al.*, 2004a, b), rainbow trout (Sakamoto *et. al.*, 2000; Nichols *et. al.*, 2003a, b), Xiphophorus sp. (Walter *et. al.*, 2004), zebra fish (Woods *et. al.*, 2000), Japanese flounder (Coimbra *et. al.*, 2003) and Nile tilapia (Kocher *et. al.*, 1998; Agresti *et. al.*, 2000). For some aquaculture species, such as rainbow trout (Sakamoto *et. al*, 2000), zebrafish (Singer *et. al.*, 2002), Japanese flounder (Coimbra *et. al.*, 2003), tilapia (Agresti *et. al.*, 2000), Arctic chaff (Woram *et. al.*, 2004) and European sea bass (Chistiakov *et. al.*, 2005), sex-specific maps have been developed.

Linkage map length differs between sexes as in species with the XY sex determination system, the female map is usually longer than the male map because of higher recombination rates in females compared to males. In zebrafish and rainbow trout, the male recombination rate close to the centromere is greatly reduced compared to the female (Sakamoto *et. al.*, 2000; Singer *et. al.*, 2002). Fishes have some of the most complex sex determination systems known in the animal kingdom (Schartl, 2004). Identification of sex-determining loci is hampered fish species due to the absence of heteromorphic sex chromosomes (Traut and Winking, 2001), variability of genetic sex determination (Volff and Schartl, 2001) and ability to switch sex depending on

the environmental conditions (Baroiller and D'Cotta, 2001). However, applying microsatellites provides a good opportunity to find a sex-determining locus due to specific features in the heterogametic sex such as an obvious reduction in recombination between markers linked to the sex-determination region in male compared to female meioses (Naruse *et. al.*, 2000) and the consistently heterozygous status of males for unique alleles in this region (Peichel *et. al.*, 2004). Using this approach, a sex-determining locus has been found in medaka *Oryzias latipes* (Naruse *et. al.*, 2000) and three-spined stickleback (Peichel *et. al.*, 2004), species without distinct sex chromosomes.

2.3.2 Individual DNA identification and parentage assignment

Microsatellites represent codominant single-locus DNA markers. For each SSR, a progeny inherits one allele from the male parent and another allele from the female parent. This simple inheritance pattern can explain the extreme popularity of polymorphic SSR loci in paternity testing. Using a panel of several microsatellite loci, a unique combined SSR genotype profile can be produced for each individual tested. The genotype profile is highly discriminating, which suggests that a random individual would have a low probability of matching a given genotype. Microsatellites extensively exploited for paternity and relatedness analysis of natural populations, hatchery broodstocks and trade control of fish products, including those from aquaculture (Liu and Cordes, 2004). Appropriate mathematical tools are available to evaluate genetic relatedness and inheritance in these systems (Luikart and England, 1999; Blouin, 2003; Jones and Ardren, 2003). An example of successful application of microsatellite markers in relatedness testing was described by Herbinger *et. al.* (1995), who analyzed a rainbow trout broodstock in a small hatchery in Canada. Using only four of five microsatellite markers, they were able to match 91% of offspring to one or two parental couples of 100 possible parental pairs and, in addition, to estimate

parental effects on progeny growth and survival. Applications of SSRs have been reported to determine paternity and reproductive contribution in wild and farmed populations of various economically significant species such as bluegill sunfish *Lepomis macrochirus* (Neff, 2001), red sea bream *Pagrus major* (Doyle *et. al.*, 2001), turbot *Scophthalmus maximus* (Castro *et. al.*, 2004), chinook salmon *Oncorhynchus tshawytscha* and rainbow trout (Bentzen *et. al.*, 2001).

Due to the small size, they are relatively stable in degraded DNA (Schneider *et. al.*, 2004) so that widely used in forensic science for individual DNA identification. An interesting example of the application of microsatellites to resolve a case of fishing tournament fraud in Finland was reported by Primmer *et. al.* (2000). In addition, microsatellite loci remain relatively stable in bone remnants and dental tissue, providing the basis for the successful application of ancient DNA for molecular analysis (Burger *et. al.*, 1999). Successful extraction and amplification of nuclear DNA from the β-globin gene region containing a polymorphic microsatellite from 12,000 year old human bone specimens has been reported (Beraud-Colomb *et. al.*, 1995). Application of microsatellites obtained from historical fish scale collections has helped to explain demographic declines in abundance, which resulted in the complete collapse of populations of lake trout in the upper Laurentian Great Lakes of North America during the past 40 years (Guinand *et. al.*, 2003). Analysis of nuclear microsatellites from ancient human and animal remnants represents an essential step to understand the genetic diversity in current populations and to provide substantial perspectives for the analysis of archeological issues, establishment of ancient baselines, heritable diseases, determination of relatedness and establishment of genealogies in prehistoric populations (Zierdt *et. al.*, 1996).

2.3.3 Phylogeny, population and conservation genetics

The molecular structure and genetic variability of microsatellites is extensively exploited in evolutionary studies of a wide variety of fish species. The vast majority of these studies attempt to infer phylogenetic relationships from microsatellite data at levels below the species level (Goldstein *et. al.*, 1999; Heath *et. al.*, 2001; Reusch *et. al.*, 2001) or for recently diverged species (McCartney *et. al.*, 2003; Stamford and Taylor, 2004), using variability within stretches of tandem repeats, which evolve significantly more rapidly than flanking regions. However, the high incidence of homoplasy (e.g., false equality of alleles based on independent mutation to the same size) with increasing evolutionary distance, may undermine the confidence of the inferred phylogenetic hypothesis, compromise the accuracy and limit the depth of phylogenetic inference (Jame and Lagoda, 1996). Another obvius problem with using SSRs for phylogenetic inference is that primers developed from one taxon may not work well on all the taxa for which genotypes are required. Although cross species ampification is common, limits on the utility of primers for amplifying homologous loci in divergent taxa are evident. Even when it is possible to amplify something in divergent taxa, the sequences may not be similar enough to permit confident assessment of orthology. Flanking regions of microsatellites have proven their value in establishing phylogenetic relationships between species and families, because they evolve much more slowly than numbers of tandem repeats. For example, a phylogeny of cichlid fishes was studied based on information from DNA sequences of the flanking region of a $(CA)_n$ microsatellite locus *TmoM27*, which showed particular conservation in several lineages of cichlids diverged more than 80-100 million years ago (Zardoya *et. al.*, 1996). Analysis revealed that the repeat region was nearly lost in the ancestor to cichlids and then amplified extensively in African taxa (Streelman *et. al.*, 1998). Indian and Malagasy cichlids formed a basal, paraphyletic group, while African and Neotropical cichlids were both

monophyletic and sister groups (Zardoya *et. al.*, 1996; Streelman *et. al.*, 1998). The authors suggested that marker *TmoM27* could be widely applied in phylogenetic studies in other perciform fishes.

Phylogeographical applications of microsatellites are eminently suitable, where population structure is observed over a large geographical scale (Koskinen *et. al*, 2002; Gum *et. al.*, 2005). The latter study on grayling *Thymallus thymallus* shows that there is strong admixture among major lineages in contact zones between drainages zones. Microsatellites are even more revealing over shorter geographical distances, where a few cases of panmixia (Dannewitz *et. al.*, 2005) and numerous cases of isolation by distance patterns (Ruzzante *et. al.*, 1999; O'Reilly *et. al.*, 2004), clinal variation (Nielsen *et. al.*, 2004), fragmentation (Lemaire *et. al.*, 2005), hybridization (Gum *et. al.*, 2005) and cryptic speciation (Fillatre *et. al.*, 2003) have been identified. In those cases, differences in the microsatellite allelic composition of populations are converted into evolutionary distances. Microsatellite genotypes are particularly helpful to detect structure in closely related populations, regardless of whether they are in evolutionary equilibrium.

SSR loci are more sensitive than allozymes for the evaluation of the dynamics of populations, including demographic bottlenecks (Spencer *et. al.*, 2000; Guinand and Scribner, 2003; Ramstad *et. al.*, 2004), population size fluctuations and effective population sizes (Gold *et. al*, 2001; Berube *et. al.*, 2002; Waples, 2002). Common measures of genetic diversity are heterozygosity (the proportion of heterozygous individuals in the population), allelic diversity (number of alleles at a locus in the population), and the proportion of polymorphic loci (Pujolar *et. al.*, 2005). Marked decreases in the observed heterozygosity and reduced number of observed alleles of tested SSRs might be attributed to the action of population genetic bottlenecks. Since they evolve 102-103 times faster than single-copy nuclear DNA, they are a powerful tool for analyzing recent and contemporary events (Ellegren, 2000).

For example, screening of microsatellites linked to the Y chromosome enabled observation of fine genetic structure of human populations as well as directions of migration and timing of post glacial human expansion in Europe (Rootsi *et. al.*, 2004). In salmonids, SSRs have been successfully used for defining temporal intervals and explaining mechanisms of severe decline of populations of brown trout in Denmark (Hansen *et. al.*, 2002) and lake trout in the North American Great Lakes (Guinand and Scribner, 2003; Guinand *et. al.*, 2003). Genome-wide scans using microsatellite markers could be applied for a search of locus-specific signatures of positive directional selection in natural populations of any species for which a high density genetic map is available (Storz, 2005). The basic strategy of how to use whole-genome screens to detect loci under positive selection was explained and referred to as hitch-hiking mapping (Harr *et. al.*, 2002). For example, a whole genome screen of DNA polymorphisms was recently performed in humans and found evidence for selective sweeps, or loci which are driven by positive adaptive selection, in non African populations (Storz *et. al.*, 2004).

Conservation and fisheries genetics focus on the effects of inbreeding, demography, contemporary genetic structuring and adaptation on the long-term survival of a species. Stock identification is a big issue (Ferguson *et. al.*, 1995), helping wildlife managers to protect biodiversity by identifying series of conservation units such as evolutionarily significant units (ESUs), management units (MUs) and action units (AUs) (Wan *et. al.*, 2004). If populations within species show significant adaptive differentiation to different habitats (ecological niches) or significant genetic differentiation, they may justify management as separate evolutionary lineages termed ESUs (Moritz, 1994). The ESU concept was developed to assign units for protection below the taxonomical level. The identification of an ESU preferably depends on significantly differentiated genetic structure detected by presumably neutral markers. In that case, SSRs represent markers of choice for identifying ESUs.

However, to find a true ESU, multiple, preferably different kinds of markers should be exploited, since size homoplasy and null (e.g., non-amplifiable) alleles could affect PCR-based microsatellite analysis (Brown *et. al.*, 2005). For example, mitochondrial and microsatellite DNA markers revealed four genetically differentiated lineages of European grayling (*Thymallus thymallus*) in central and northern Europe, which evolved in geographical isolation during the Pleistocene and could be recognized as the ESUs (Gum *et. al.*, 2005). Genetic analyses often reveal differences between sampled populations with substantial but no complete phylogenetic separation, which have minor but statistically significant differences in allele frequency of nuclear or mitochondrial loci. These populations are termed MUs (Wan *et. al.*, 2004). The MU is considered to be a conservation unit level below that of the ESU, which is based on multiple evidence such as molecular markers, habitat use and adaptive characters. AUs display genetic patterns of living populations. Microsatellites could be more successfully applied for identifying MUs and AUs than mitochondrial DNA markers, since mitochondrial DNA has maternal inheritance. Conservation strategies depend on neither paternal nor maternal variation, but focus on using biparental polymorphism of nuclear DNA to reflect characteristics needed to cope with environmental conditions (Zhang and Hewitt, 2003). A high mutation rate of SSR loci also supports use of these markers in the genetic analysis of very recent events in the dynamics of populations, e.g., to MUs and AUs.

2.3.4 Molecular epidemiology and pathology

Genomic instability of microsatellite has been extensively evaluated in the field of carcinogenesis, where chromosomal rearrangements (e.g., translocations, insertions and deletions of genomic regions) occur (Charames and Bapat, 2003). Carcinogenic events often happen within a genomic region harboring a tumor suppressor gene and hence inactivate the gene (Grady,

2004). Carcinogenic rearrangements are associated with loss of heterozygosity (LOH) in microsatellites located within the affected chromosome region. Thus, detecting microsatellite LOH in tumor tissues contributes not only to molecular diagnosis of cancer, but also points the possible location of a tumor suppressor gene (Presneau *et. al.*, 2003). The instability of triplet motifs was found in lower vertebrates, including fishes (Schartl *et. al.*, 1998; Liu *et. al.*, 2001). A variable number of trinucleotide repeats occurred within the coding region of functionally important genes expressed in the brain of adult fishes such as the channel catfish orthologue of the *RAD23B* gene (Liu *et. al.*, 2001) and the zebrafish *Clock* gene (Saleem *et. al.*, 2001). A *RAD23B* gene product functions in the nucleotide exclusion repair (NER) pathway. NER defects are associated with higher incidence of mutagenesis and carcinogenesis and cause Xeroderma pigmentosum, an autosomal recessive disease in humans (Sancar and Hearst, 1993). The Clock locus regulating circadian rhythms is highly conserved in various organisms. Alterations of circadian rhythms could be related to a large number of diseases, including psychiatric disorders in humans (King and Takahashi, 2000). The polyglutamine $(CAG)_n$ tract at the Clock gene is highly polymorphic in Drosophila, ranging from 25 to 33 pure glutamine repeats. In zebrafish, the $(CAG)_n$ stretch includes up to 51 repeat units (Saleem *et. al.*, 2001). However, it is shortest and non-polymorphic in human. The lack of polymorphism may indicate that variation at this locus is deleterious to the individual and hence not tolerated.

Microsatellite based screening strategies can be used in the fields of veterinary and medical parasitology and for molecular studies of infectious diseases. This includes mapping and further identification of genes responsible for resistance to parasites and pathogens and the identification of genes controlling drug resistance in pathogenic organisms (Gasser, 1999; Naidoo and Chetty, 1998; Behnke *et. al.*, 2003; Anderson, 2004). Such approaches have been applied in a variety of domesticated and farmed animals and plants

(Naidoo and Chetty, 1998; Yencho *et. al.*, 2000). In farmed fishes, they have been mostly performed on Salmonidae. In rainbow trout, genomic DNA was screened for loci controlling natural killer cell-like activity (Zimmerman *et. al.*, 2004) and linked to resistance to infectious hematopoietic necrosis virus (Palti *et. al.*, 1999; Khoo *et. al.*, 2004), ceratomyxosis (Nichols *et. al.*, 2003b) and pancreatic necrosis virus (Ozaki *et. al.*, 2001; Gibson, 2002). Identification of six microsatellite loci linked to resistance to red sea bream iridovirus, which causes high mortalities in cultured red sea bream in Japan, have also been reported (Inami *et. al.*, 2005).

2.3.5 Quantitative trait loci mapping

A quantitative trait is one that has measurable phenotypic variation owing to genetic and/or environmental influences. The variation can be measured numerically (for example, height, size or blood pressure) and quantified. Generally, quantitative traits are complex (multifactorial) and influenced by several polymorphic genes and by environmental conditions. A QTL is a genetic locus (gene), the alleles of which affect phenotypic variation. One or many QTLs can contribute to a trait or a phenotype. When more than one QTL influences a particular trait, each might have a different effect size, and the effects of individual QTLs can vary from strong to weak. The size and nature of these effects also can be affected by the genetic back ground (the total genotype of the individual), and interactions between QTLs are common (Mackay, 2001) To date, microsatellite based strategies (scans across individual chromosomes and a whole genome) represent appropriate techniques to identify QTLs, particularly those that are associated with medically, economically and evolutionarily important complex traits. Due to the genome-wide distribution and high levels of allelic polymorphism, SSR loci are very helpful in coarse and fine linkage mapping approaches. Coarse

mapping resolves detection of a putative QTL in a chromosomal region, usually within a range of 10-30 cM. For a given QTL, the likelihood of success and mapping resolution depends on the number of loci screened and the magnitude of their effect on the trait of interest. Also important are recombination events in the mapping population, the mode of expression of the trait (dominant, recessive or additive), size of the mapping population, and number of genes that define the quantitative trait (Glazier *et. al.*, 2002).

Characteristics and types of experimental crosses used to breed a mapping population are also crucial in precise QTL mapping. For farmed fishes, the first mapping of an economically important QTL was reported in 1998 (Jackson *et. al.*, 1998). To date, no QTL gene has been defined, but several microsatellite based QTL screenings have been performed. Most of these mapping experiments have targeted three salmonid species (Atlantic salmon, rainbow trout and Arctic chaff). These screenings include searches for QTLs related to temperature tolerance (Jackson *et. al.*, 1998; Danzmann *et. al.*, 1999; Perry *et. al.*, 2001; Cnaani *et. al.*, 2003; Somorjai *et. al.*, 2003), body weight (Cnaani *et. al.*, 2003; O'Malley *et. al.*, 2003; Borrell *et. al.*, 2004; Reid *et. al.*, 2005), body length (Borrell *et. al.*, 2004), spawning date (Sakamoto *et. al.*, 1999; O'Malley *et. al.*, 2003), embryonic development rate (Nichols *et. al.*, 2000; Robison *et. al.*, 2001) and condition factor (Nakamura *et. al.*, 2001; Perry *et. al.*, 2003; Reid *et. al.*, 2005). In rainbow trout, the sex-linked microsatellite marker *OmyFGT19TUF* showed significant association with fork length (FL) and upper thermal tolerance (UTT), explaining dependence of male advantages in FL and UTT compared to their female sibs with the origin of the Y chromosome (Perry *et. al.*, 2005). QTL mapping in natural populations represents a powerful tool to study the genetic architecture of fitness traits and reproductive isolation. This approach has not been extensively exploited yet since there are no well developed genetic and genomic tools for

most free living species. The search for QTLs in wild populations requires the design or observation of appropriate crosses to create a suitable mapping population, which contains individuals of measured phenotype and which can be pedigreed. The availability of a genetic map of variable markers is also crucial (Slate, 2005), Marine three spined stickleback represents a good example of an organism that successfully colonized new freshwater lakes approximately 15,000 years ago and rapidly adapted to a diverse array of new environments. Within these lakes, sympatric forms adapted to a different ecological niche benthic forms have reduced body armour, increased body length and decreased number of gill rakers, while limnetic forms more closely resemble marine forms with a streamlined body, extensive armour and a large number of gill rakers. To dissect the genetic architecture of the traits that cause reproductive isolation, a mapping population was derived from a benthic female and lirnnetic male from Priest Lake (British Columbia), followed by backcrossing an F1 male to a benthic female (Peichel *et. al.*, 2001). A medium-density linkage map containing 227 microsatellites was constructed. The F2 progeny (n=92) was genotyped to find QTLs linked to several gill raker and body annour traits. A major QTL was mapped for every trait, each explaining between 17% and 37% of the phenotypic variance. A new and relatively large (360 F2 progeny) stickleback mapping population was recently produced using a marine female (from Japan) and benthic male (from Lake Paxton, British Columbia) to study the genetics of armour plate reduction (Colosimo *et. al.*, 2004). QTLs were discovered for lateral plate number, with a major locus in linkage group 4 accounting for 75% of the phenotypic variance. A second cross was generated from benthic and limnetic fish (Lake Frient, California). Three of the QTLs (including the major one) segregated in the second population (Colosimo *et. al.*, 2004). The authors also performed a complementation cross between Paxton and Frient populations and found that the same gene within the major QTL was responsible for the high plate number

in these populations. This indicates that a single gene causing a major shift in phenotype can explain cases of parallel evolution (Colosimo *et. al.*, 2004)

2.3.6 Marker-assisted selection

Marker-assisted selection is based on the concept that it is possible to infer the presence of a gene from the presence of a marker tightly linked to the gene. For this purpose, it is important to have high-density and high-resolution genetic maps, which are saturated by markers in the vicinity of a target locus (gene) that will be selected. Strategies to find markers tightly linked to the target gene are similar to those that are used for fine QTL mapping. Strategies, such as flanking marker analysis (Dixon *et. al.*, 1995) and pooled sample mapping (Churchill *et. al.*, 1993), are used to order these markers. Once a tight linkage is found between a molecular marker and a gene of interest, the inheritance of the gene can be traced in breeding programs. Successful implementation of MAS requires well developed genomic tools, including optional information on genetic variations relevant to the QTL phenotype, mode of inheritance, interactions with other contributing QTLs and economical magnitude of the QTL studied (Poompuang and Hallerman, 1997). To plan MAS, breeders also should take into account possible interactions between QTLs, which could relate to each other and have overlapping genetic backgrounds. In that case, MAS should preferably represent a complex selection index and take into consideration all economically significant traits that interact.

In fish culture, DNA marker-based techniques have been applied in several cases, for example, in breeding programmes for Atlantic halibut *Hippoglossus hippoglossus* (Jackson *et. al.*, 2003), channel catfish (Waldbieser and Wolters, 1999), European sea bass (Garcia de Leon *et. al.*, 1995), Japanese flounder (Hara and Sekino, 2003; Sekino *et. al.*, 2003) and salmonids (Herbinger *et. al.*, 1995; Fjalestad *et. al.*, 2003; Wilson *et. al.*, 2003).

Apart from facilitating survival under variable environmental conditions, a higher genetic diversity within a progeny array stemming from multiple matings by females might also serve to reduce the potential cost of inbreeding and reduce the deleterious effects of genetic incompatibility between two partners (Jennions and Petrie, 2000). Microsatellite markers are useful in early stages of MAS for the primary selection of parents for further crossing and subsequent genetic characterization of progeny. For this, SSRs linked to the target QTL would be used. Further improvements, such as enrichment of linkage maps with type I markers, construction of high-resolution linkage maps, development of physical maps and their integration with linkage maps, fine QTL mapping using the candidate gene approach, will lead to the replacement of SSRs by other types of genetic markers (ESTs and SNPs) in later stages of marker-assisted breeding programmes. This would lead to even more precise selection by gene assisted selection (GAS), based on the use of favorable haplotyoe and genotypes derived from genes directly contributing to the target trait (Hulata, 2001).

Materials and Methods

3. MATERIALS AND METHODS

3.1 Collection of fish samples

3.1.1 Fish samples and sites of collection

Chitala chitala specimens were collected during the present study (photograph. 2) through commercial riverine catches from three riverine locations in different geographical areas in India i.e. Satluj (lat. 31° 09' N; lon. 74° 56' E; n=16), Brahmaputra (lat. 26° 16' N; lon. 91° 46' E; n=15) and Bhagirathi (lat. 24° 05' N; lon. 88° 06' E; n=15). The riverine locations were chosen to cover geographically distant populations of *C. chitala* (fig. 1). River Satluj is a part of Indus river system, whereas Brahmaputra belongs to Ganga basin (ECAFE, 1966). The Ganga developed in the front of the Himalayas after Gondwanaland dashed against Eurasia plate. All the rivers i.e. Satluj, Yamuna, Bhagirathi etc, appeared over the course as lateral rivers. The samples (n=46) were obtained during May 2003 to Sep 2006. Total length/body weight ranged from 70 cm/3.5 kg to 25 cm/250 g. The sample size, location and time of collections of *C. chitala* are given in table. 1.

3.1.2. Blood and muscle samples

The blood samples was collected through caudal puncture with the syringe washed with heparin and fixed in 95 % ethanol at 1: 5 (blood: ethanol) ratio. A small piece of white muscle was taken from below the skin and above the lateral line and sample fixed in 95% ethanol. Caution was taken to avoid any red muscle. The blood and muscle samples in 95% ethanol were transported from the field at room temperature and stored in the laboratory at 4°C.

Photograph 2. *Chitala chitala* blood samples collection from natural populations.

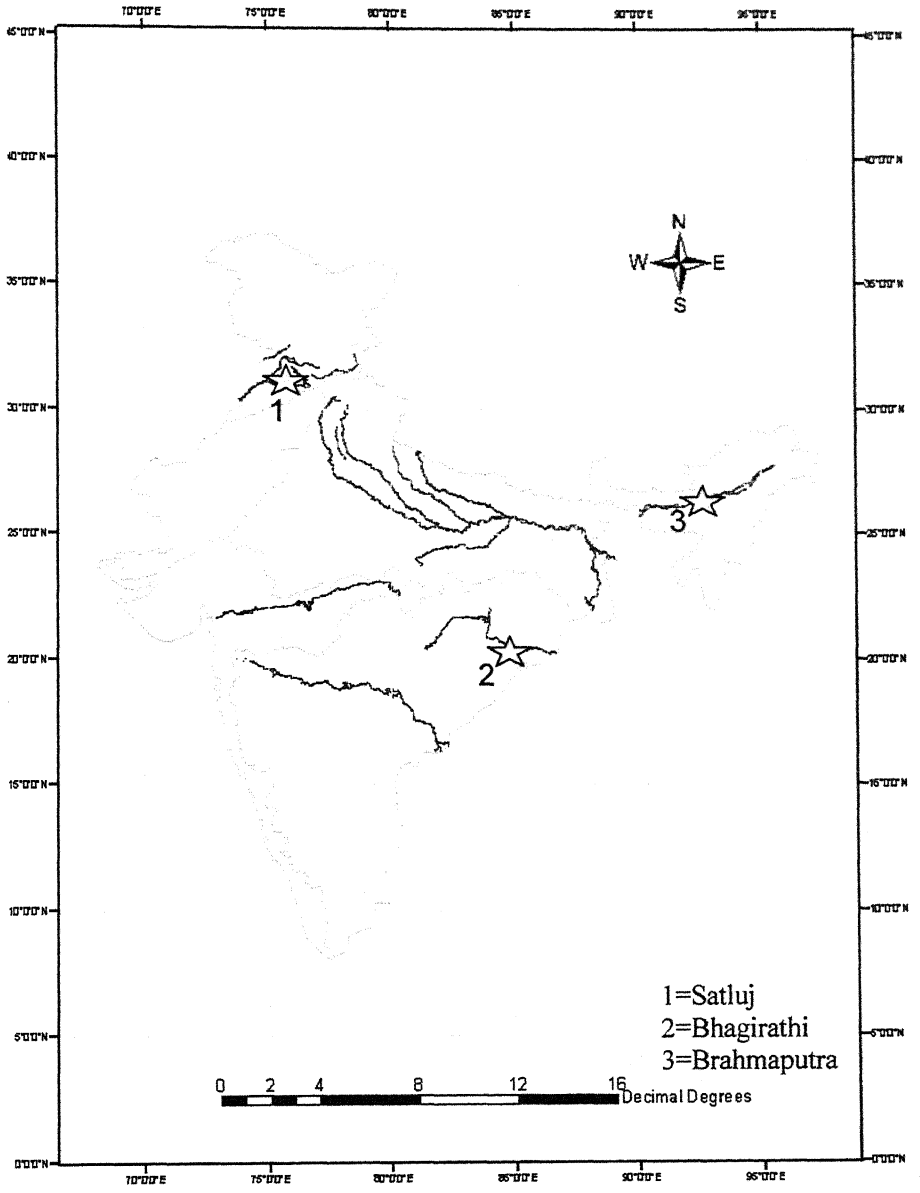


Fig. 1. Collection sites of *Chitala chitala* samples during present study.

Table.1. The number and location of collection site of *Chitala chitala* samples from different rivers.

River system	River	Location/Position	Year of Sampling	Total samples
Indus	Satluj	Hari Ke Pattan, Dist. Amritsar, Punjab (31° 09' N; 74° 56' E)	May,2003 Sep,2006	16
Ganga	Bhagirathi	Farakka, Dist. Murshidabad, West Bengal (24° 05' N; 88° 06' E)	July, 2003 May, 2004	15
Brahmaputra	Brahmaputra	Kalang, Assam (26° 16' N; 91° 46' E)	July, 2005	15
			Total	46

3.2 Isolation and quantification of genomic DNA

Total genomic DNA from ethanol preserved blood was extracted by the modified procedure described by Ruzzante *et. al.* (1996). Approximately 50 µl of ethanol fixed blood cells were washed twice with High TE buffer (100mM Tris. HCl, 40 mM EDTA, pH 8.0) and incubated overnight in 0.5 ml of lysis buffer (10 mM Tris. HCl, 1 mM EDTA, 400 mM NaCl, pH 8.0), containing 1% sodium dodecyl sulphate and 0.2 mg/ml proteinase K, at 37°C. The DNA then was purified by extracting the incubation mixture once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) and was precipitated with 2 volume of ice-cold absolute ethanol. After washing with 70% ethanol, the pellet was dried and resuspended in TE, pH 8.0 (10 mM Tris-HCl; 0.1 mM EDTA). 1µl of RNAase (Appendix. II.5) was added in each sample tube and samples were kept at 37°C in water bath for 2 hours.

Concentration of DNA was determined through 0.7% agarose gel electrophoresis with ethidium bromide incorporated and 0.5X TAE (1X= 40 mM Tris acetate, 1 mM EDTA) as running buffer. The gels were run at 10 V/cm and visualized under UV transilluminator. The concentration of total DNA was determined by comparing with the known quantity of DNA and adjusted to 200 ng/µl and stored at 4°C.

3.3 Construction of microsatellite enriched genomic library

Microsatellite enriched genomic library was constructed following the method of Fleischer and Loew (1995) and M, Hamilton and R, Fleischer (personal communication) as below.

3.3.1 Digestion and dephosphorylation of genomic DNA

5 µg of genomic DNA was digested with 30 units of *Sau*3A1 restriction enzyme in 50 µl reaction at 37°C over night according to manufacturer

instruction (MBI Fermentas). Digested product was then checked on 1% agarose for complete digestion. To prevent self-ligation 5' end of digested DNA fragments dephosphorylated using 1 unit of CIAP enzyme at 37°C for 2 hours followed by 20 minutes incubation at 65°C to inactivate CIAP enzyme.

3.3.2 Size selection and extraction of DNA fragments

Digested fragments were run on 1% low melting agarose along with 100 bp ladder. Size selection of digested gnomic fragments was carried out by gel extraction kit (Quigen). DNA fragments ranging from 300 to 800 bp were excised from gel and suspended QG buffer (1:3 ratio of gel weight to buffer volume) and incubated at 50°C until agarose gel was completely melted than after mixing with one volume of isopropyl (weight/volume) were passed through the column (provided with kit). The column was washed twice with 0.75 ml of PE buffer. DNA was eluted from the column by applying 20 µl of elution buffer and centrifuged briefly and quantified on 1% agarose gel.

3.3.3 Preparation of SAU linkers

SAULA (5'-GCG GTA CCC GGG AAG CTT GG-3') and SAULB (5'-GAT CCC AAG CTT CCC GGG TAC CGC-3') oligonucleotides were used to synthesize complementary linkers. 1200 pmoles of SAULB linker was phosphorylated using 10 µl of 10X kinase buffer, 1 unit of T4 Polynucleotide Kinase enzyme (MBI Fermentas) and 10 mM ATP in a 100 µl reaction cocktail and then incubated at 37°C for 2 hours followed by heating at 65°C to inactivate enzyme. Double stranded linkers were prepared using 600 pmoles of SAULA and phosphorylated SAULB linkers in a 200 µl reaction and left at room temperature for 10 minutes.

3.3.4 Ligation of linkers to size selected genomic DNA fragments

Eluted fragments (step 3.3.2) and prepared linkers were (step 3.3.3) heated at 65°C for 10 minutes to melt sticky ends and then placed at ice. 1 µg

(6.154 pmoles of end genomic DNA) of insert DNA and 6.154 pmoles of linkers were ligated using, 2 µl of 10X ligase, 5 units of T4 DNA ligase in a 20 µl reaction at 16°C for overnight. Ligation product was then checked on 1% agrose gel for successful ligation.

3.3.5 Amplification and purification of linker ligated inserts

Genomic fragment were amplified in 50 µl reaction mixture contained 1X PCR buffer (10 mM Tris-HCl, pH 9.0 50 mM KCl; 0.01%gelatin), 12.5 mM of $MgCl_2$, 2.5 mM of dNTP, 5 µl (10µM stock) of SAULA primer and 1.5 units of *Taq* polymerase and 50 ng of template DNA (from step 3.3.4).

Reaction conditions

Reaction conditions for amplification of genomic fragments are as follows.

Sl. No.	Steps	Conditions		No. of Cycles
1	Initial denaturation	94°C for 5 minutes	}	1 cycle
2	Denaturation	94°C for 40 seconds	}	
	Annealing	60oC for 30 seconds		35 cycles
	Elongation	72°C for 2 minute		
3	Final elongation	72° C for 4 minutes	}	1 cycle
4	Soaking	4° C		

Amplification was performed in MJ Research PTC 200 thermocycler, the PCR products were stored at 4°C. 3 µl of amplified inserts were checked on 1 % agrose for successful amplification.

PCR product was purified by adding 1/10 volume (4.7 µl) of 3 M sodium acetate and 2 volume (100 µl) of ice cold absolute ethanol. Reaction was incubated in deep freezer (-20°C) for 30 min followed by centrifugation at

12,000 rpm for 10 minutes. Supernatant was discarded and pellete again washed with 70% ethanol and again centrifuged for 5 minutes. DNA pellete air dried for 10 minutes and dissolved in 25 µl of TE buffer. Purified product was checked and quantify on 1% gel and was used as probes for the hybridization (step 3.3.7).

3.3.6 Construction of microsatellite repeats (concatmers) by ligation and amplification

Long sequences of microsatellite repeats were constructed as concatmers and used to select large microsatellite repeat arrays from the amplified DNA fragments. Complementary pairs of oligonucleotides probes were used for the concatemeriztion (table. 2). Each 20 µg oligonucleotide probes of complementary pairs were phosphorylated using 10 units of T4 Polynucleotide Kinase and 1 mM ATP in a 50 µl reaction. Reaction cocktail was incubated at 37°C for 2 hours followed by heating at 65°C to the inactivate enzyme. 5 µg of phosphorylated complementry pairs of oligonucleotide was ligated in 20 µl reaction using 5 units of T4 DNA ligase and 1 mM of ATP followed by over night incubation at 16°C. 3 µl ligated product checked on 1% agarose for successful concatmerization.

To further increase the size of the oligonucleotide concatemer self priming PCR was done using 2 µl of concatemerized oligonucleotide in 50 µl reaction mixture having same content has in step 3.2.5 except the primer. Thermal profile include: [40 cycles (94°C for 1 min, 65°C for 1 minute; and 72°C for 2 minutes) and final extension 4 minutes at 72°C]. Amplified product purified as same in step 3.3.5 and checked on 1.5% agarose gel along with 100 bp ladder document the size and relative amount of the product.

Table.2. Probes used for microsatellite enriched genomic library construction of *Chitala chitala.*

Sl. No	Oligonucleotide 1	Oligonucleotide 2
1.	$(GT)_{10}$	$(CA)_{10}$
2.	$(GA)_{10}$	$(CT)_{10}$
3.	$(CAC)_{10}$	$(GTG)_{10}$
4.	$(AGA)_{10}$	$(TCT)_{10}$
5.	$(TAT)_{10}$	$(ATA)_{10}$
6.	$(CTT)_{10}$	$(GAA)_{10}$
7.	$(CCT)_{10}$	$(GGA)_{10}$
8.	$(GGC)_{10}$	$(CCG)_{10}$
9.	$(CTG)_{10}$	$(GAC)_{10}$
10.	$(CTC)_{10}$	$(GAG)_{8}$
11.	$(GACA)_{8}$	$(CTGT)_{8}$
12.	$(GCAC)_{8}$	$(CGTG)_{8}$
13.	$(GATA)_{8}$	$(CTAT)_{8}$
14.	$(CAGA)_{8}$	$(GTCT)_{8}$

3.3.7 Hybridization of amplified inserts to amplified repeats.

Selections of DNA fragments that contain microsatellites were accomplished by hybridization of the amplified DNA fragments (step 3.3.5) to amplified repeats (step 3.3.6) bounded to nylon filters. 1 µg of amplified repeats were heat denaturated and spotted on 1 cm^2 6X SSC soaked pieces of girded membrane and labeled each gird (DNA side up) with repeat sequence. Membranes were then immobilized in UV crosslinker at optimal intensity mode (1200 µJ/cm^2) for 1 minute. Membranes were then rinsed with denaturing solution (50 mM KOH/0.01% SDS) and then with 6X SSC to remove any unbounded oligonucleotide from the membrane. Membranes were prewetted in DDW for 10 min and transferred to 1.5 ml screw-top microfuge tube DNA side toward the inside of the tube. Each membrane prehybridized in 1ml of hybridization buffer (Roche) at 50°C for dinucleotide and 60°C for tri and tetranucleotid repeats in hybridization oven with gentle agitation for 1 hour. Prehybridization solution poured out and replaced with 100 µl of the same preheated hybridization solution. 1 µg of amplified fragments denatured by heating to 95°C for 5 minutes and then added full directly to hybridization solution while hot and mixed with pipettor. Tubes were placed in rotating canister of a hybridization oven and incubated for overnight at 50°C for dinucleotide and 60°C for tri and tetranucleotide repeats. Membranes were then washed with washing buffer (2.0 X SSC, 0.1% SDS) to remove unbound DNA for 15 minutes at room temp and 30 minutes at hybridization temperature.

3.3.8 Extraction and amplification of bound DNA from membrane

Membranes were then transferred to 1.5 ml eppendof tubes and 100 µl of denaturing solution (50 mM KOH/0.01% SDS) was added in tubes. The denaturing solution was pipetted over the filters 5-10 times at room temperature and then left for 5 minutes. Membranes were transferred to new eppendof tubes

and 100 µl of neutralizing solution (50 mM Tris-HCl, pH 7.5/0.01% SDS) was added in tubes and solution was pipetted over the filters 5-10 times and then left in solution for 5 minutes. Both denaturing and neutralizing solutions were combined in single tube and mixed with 1/10 volume of 3.0 M sodium acetate (pH 8.0), 2 µl of 100 µM SAULA primer and 2 volumes ice-cold absolute ethanol. Tubes were placed in ultra-cold freezer (-80°C) for 30 minutes. Tubes were then centrifuged at 12000 rpm at 4°C for 10 minutes. Supernatant was discarded and the pellete was rinsed in 400 µl of 70% ethanol. Supernatant was again discarded and pellete resuspended in 20 µl of TE buffer. Extracted DNA fragments reamplified in a 50 µl PCR reaction with the SAULA primer as in step 3.3.5 except that 5 µl of post-hybridizaed eluted DNA was used. Amplified product purified as same in step 3.3.5 and checked on 1.5% agarose for amplification.

3.3.9 Digestion, purification and phosphorylation of amplified DNA fragments

Linkers were removed from 30 µl (10 ng) of amplified hybridized fragments using, 5 µl of 10X buffer and 20 units of *Sau*3A1 restriction enzyme in 50 µl reaction at 37°C for 2 hours. DNA fragments were purified from digested linkers using QUIGEN purification kit. Digested inserts mixed in DNA solution I and then passes through the column (provided with kit). The column was washed twice with 0.75 ml of buffer PE. DNA was eluted from the column by applying 20 µl of elution buffer and centrifuged briefly. Eluted DNA inserts (fragments ready to clone) quantified on 1% agarose gel and were phosphorylated using 10 units of T4 Polynucleotide Kinase and 1 mM ATP in a 50 µl reaction for 1 hour at 37°C.

3.3.10 Preparation of Vector (pUC18)

10 µg of pUC18 vector was digested with 5 µl of 10X buffer and 15 units of *Bam*H1 enzyme (MBI Fermentas) in a 50 µl reaction at 37°C for 2 hours. Digested product was checked on 1% agarose for successful digestion along with size standard (λ *Hind*III/*Eco*RI) and undigested vector. Digested product was purified by ethanol precipitation as in step 3.3.5 and dissolved in 20 µl of TE buffer. The phosphate group removed from digested plasmid using 1 unit CIAP enzyme to prevent self ligation as per instruction provided by manufacturer (MBI Fermentas) in a 30 µl reaction at 37°C for 2 hours.

3.3.11 Ligation of Vector and inserts

50 ng of digested plasmid ligated with 100 ng of prepared inserts in a 20 µl reaction by using, 1X ligation buffer, 100 mM of ATP and 5 units of T4 DNA ligase. Reaction cocktail was then incubated in water bath at 16°C for overnight and stored in -20°C until transformation.

3.3.12 Preparation of competent *E. coli* (DH5α) cells

Fresh competent cells were prepared using calcium chloride method (Sambrook *et. al.*,1989). DH5α cells were taken from stock and inoculated in 3 ml 2X YT (Appendix. V.1) broth. Cells were cultured at 37°C and 250 rpm for overnight. 1 ml of cultured broth was inoculated in 100ml of fresh 2X YT broths. Culture was grown at 37°C and 250 rpm till O.D of culture reached 0.3-0.4 at 600 nm. Culture was transferred aseptically to sterile, disposable, ice-cold 50 ml polypropylene tubes (Falcon 2070) and chilled on ice for 10 minutes. Cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C and media was discarded the from the cell pellets. Cell pellet was resuspened in 10 ml of ice-cooled 0.1 M $CaCl_2$ and stored on ice for 15 minutes. Cells were again centrifuged at 4000 rpm for 10 minutes at 4°C. Supernatant was discarded and pellet resuspended each pellet in 4 ml of ice-

cooled 0.1 M $CaCl_2$. Competent cells stored at 4°C for overnight before transformation.

3.3.13 Transformation of competent cells with ligation mixture

Transformation was performed using heat shock procedure (Sambrook *et. al.*, 1989). 10 µl (~50 ng) of ligation mixture was added directly to 200 µl of competent cell in a 1.5 ml tubes and incubated on ice for 30 minutes. A heat shock of 90 seconds was given by transferring the tubes to 42°C water bath and immediately putting back on ice for two minutes. 800 µl of 2X YT were added to the tube and incubated at 37°C in a shaker incubator at 200 rpm. After an hour of incubation, 200 µl of culture was plated on YT agar plates containing ampilicilin, X Gal and IPTG (Appendix. V) and incubated at 37°C for overnight. Plates were observed next day for the presence of blue and white colonies.

3.4 Screening of microsatellite enriched genomic library

3.4.1 Labeling of Probes

Di, tri and tetra nucleotide listed in table 2 labeled with DIG using labeling and detection kit (Roche). 3 µg of probes were denatured by heating for 10 min at 95°C and hexanucleotide mix (2 µl), dNTP mixture (2 µl) and 1 µg of Klenow fragment added directly on ice. Reaction cocktail incubated at 37°C for overnight. 2 µl of 0.2 M EDTA was added to stop reaction and labeled DNA precipitated by adding 2.5µl 4 M LiCl and 75 µl of ice cold 100% ethanol followed by 30 minutes incubation at -70°C. DNA was recovered by centrifugation at 12000 rpm for 15 minutes. Pellet was washed with 50 µl of 70% ethanol and after air drying dissolved in 50 µl TE buffer.

3.4.2 Screening of colonies with labeled probes

90 mm^2 positively charged nylon membrane was used and replica was prepared according the method suggested in Sambrook *et. al.* (1989). Membranes were cut at one corner and placed over colonies (master plate) for 2 minutes. To align the membrane with colonies holes was made in membrane and media. Membrane was peeled and placed in fresh culture (replica) plate with colony side down for 2 minutes and holes were made to align. After lifting membranes both master and replica plates were incubated at 37°C for 6 hours. 4 circular Whatman 3 MM filter papers were cut and saturated with SDS, Denaturing solution, Neutralizing solution, 2X SSC respectively (Appendix. III.4). Membranes were exposed to above filter in same sequence for 5 minutes in each filter with colony side up and finally air dried for 30 minutes at room temp. DNA was crossed linked over membranes in UV cross linker at optimal intensity mode (1200 $\mu J/cm^2$) for 1 minute.

3.4.3 Hybridization and detection positive clones on nylon membranes

Membranes were prehybridized in 20 ml standard hybridization buffer at 50°C for dinucleotide and 60°C for tri and tetranucleotid repeats in a hybridization oven. After 1 hour of incubation hybridization solution was replaced with 2.5 ml fresh hybridization solution having denatured probes (16 ng/ml) and incubated at hybridization temperature (50°C for dinucleotide and 60°C for tri and tetranucleotid repeats) for overnight. Membranes were washed twice in ample of 2X SSC, 1% SDS at room temperature followed by twice washes in 0.1X SSC, 0.1% SDS at hybridization temperature for 15 minutes. Membrane was rinsed briefly for 5 minutes in washing buffer (Appendix. IV) and incubated for 30 min in blocking solution (1X). Membrane was incubated in 20 ml Anti-DIG –AP conjugate diluted to 1:10000 in blocking solution for 30 minutes followed by two washes with 50 ml washing buffer. Membrane was equilibrated in 15 ml of detection buffer for 5 minutes. Detection buffer

was then poured off and membrane was incubated in 10 ml freshly prepared color-substrate solution for overnight. The reaction was stopped by washing the membrane for 5 minutes with 50 ml of DDW and the membrane was observed for positive signal and matched with master plate to detect the colonies producing color.

3.5 Isolation of microsatellite sequences and primer designing

3.5.1 Plasmid isolation

Colonies were picked and inoculated in 3 ml of 2X YT broth containing ampicilin followed by overnight incubation at 37°C. Culture was pelleted in 1.5 ml tubes at 10000 rpm for 1 minute and plasmid was isolated manually according to Sambrook *et. al.* (1989) and by kit method (Quigen) for final sequencing. In former method pellet was washed with 500 µl of STE buffer (Appendix. VI) and again centrifuged. Pellet was resuspended in 100 µl of solution I by vortexing. 200 µl freshly prepared solution II was added and content mixed by inverting tubes six times. Tubes were stored on ice for 5 minutes. 150 µl of solution III added to tubes again mixed by inverting tubes and incubated on ice for 5 minutes. Tubes were centrifuged at 10000 rpm for 10 minutes at 4°C and supernatants were transferred to fresh tubes. Twice volume of absolute ethanol added and the pellet was recovered by centrifugation. Pellet was washed with 70% ethanol and air dried for 15 minutes. Pellet was dissolved in 50 µl of TE buffer containing DNAase free pancreatic RNAase (20 µg/ml) and 2 µl of which checked on 1% agarose gel for successful isolation and quantification.

3.5.2 Sequencing of plasmids

Plasmids were sequenced using Big Dye terminator kit (Amersam Biosciences) and the reaction consisted of 5 pmoles M13 forward primer, 4 µl

sequencing premix (supplied with kit) and 200 ng plasmid in 10 µl reaction mixture. Thermal profile includes

Sl. No.	Steps	Conditions		No. of Cycles
1.	Denaturation	94°C for 30 seconds	}	
2.	Annealing	50°C for 20 seconds		25 cycles
3.	Elongation	60°C for 1 minute		
4	Soaking	4° C		Forever

Sequenced product was purified by adding 1 µl of 7.5 M ammonium acetate and 27.5 µl of absolute ethanol to each tube. Reaction was incubated at ice for 15 min precipitated by centrifugation at 12000 rpm for 15 minutes. Pellet was washed with 200 µl of 70% ethanol and vaccume dried for 5 minutes. Pellet was dissolved in 10 µl of Megabase loading solution and loaded in sequencing plates. Sequencing was done on Amersam Biosciences MEGABASE automated machine. Samples were injected in the capillaries filled with matrix at 3 KV for 60 second and run at 9 KV for 180 minute.

3.5.3 Primer Designing

The primers for microsatellite sequences were designed on the basis of their melting temperature, secondary structure, sequence homology between the forward and reverse primers, G/C contents, through software Primer Select Ver. 5.07 (DNA Star) and custom synthesized for use.

3.6 Characterization of isolated microsatellite loci

Designed primers were tested through PCR and PAGE and 46 individuals belonging to different river system Satluj (n=16), Bhagirathi (n=15) and Brahmaputra (n=15) were used for genetic variation studies.

3.6.1 Amplification Reaction

For PCR of microsatellite loci, 50 ng of total DNA was used as template DNA, in a total reaction volume of 25 µl. The amplification reaction mixture contained 1X PCR buffer (10 mM Tris-HCl, pH 9.0 50 mM KCl; 0.01%gelatin), 2.0 mM of $MgCl_2$, 0.2 mM of dNTP, 5 pmoles of forward and reverse primer each and 1.5 units of *Taq* polymerase and template DNA. Reaction conditions for amplification of microsatellite loci are given below. The annealing temperature (T_a) was standardised 3-10°C below melting temperature (T_m) for amplification of scorable bands. Amplification was performed in MJ Research PTC 200 thermocycler, the PCR products were stored at 4°C and analyzed within 24 hours.

Sl. No.	Steps	Conditions		No. of Cycles
1	Initial denaturation	94°C for 5 minutes	}	1 cycle
2	Denaturation	94°C for 30 seconds	}	
	Annealing	T_a* for 30 seconds		25 cycles
	Elongation	72°C for 1 minute		
3	Final elongation	72° C for 4 minutes	}	1 cycle
4	Soaking	4° C		

* Annealing temperature for each primer given in table. 5.

3.6.2 Polyacrylamide gel electrophoresis (PAGE) and detection of PCR products

Glass plates, alumina plates, spacers and combs were first cleaned with water and then with methanol and units assembled (Hoeffer Mighty small unit) according to manufacturer instructions on a leveling table. Dissolved agarose solution (1%) was poured between plates for sealing of unit. Depending on size of amplified product 6-12% non-denaturing polyacrylamide solution (Appendix. VII.6) gels polyacrylamide was poured between plates of casting unit. 8 µl of PCR product was mixed with 2 µl of 1X bromophenol blue and was loaded in the gels. The DNA size markers (*Msp*I cut pBR322 DNA) were run on both sides and middle of the gel to determine the size of the amplified products. The gels were run for 5 hours at 15 V/cm at 4-6°C. The bands were visualized by staining with silver stain (Silver Staining Kit, Amersham Pharmacia Biotech USA)

3.6.3 Assigning of alleles and genotyping

Molecular weights of the PCR products were calculated in reference to the standard molecular weight markers with the software BIOVIS 1D4. The bands having the same molecular weight were taken as the same allele in different individuals. The alleles were designated according to their molecular weight. An individual is genotyped as homozygote or heterozygote according to the number and molecular weight of the allele(s) present in that particular individual. The genotypes of all the samples were recorded on MS Excel sheets. The data were initially entered according to their loading sequence on gels and then it was sorted so that the data were population wise with the genotype of all the loci in their respective columns.

3.7 Statistical analysis of data

To analyze variation at microsatellite loci, allele frequencies at each locus were calculated with software GENETIX ver. 4.05 (Belkhir *et. al.*, 1997). A locus was considered to be polymorphic when frequency of the most common allele is equal to or less than 0.99 (Nei, 1975). Observed and expected heterozygosities, percent polymorphic loci and mean number of alleles per locus were calculated with GENETIX. The Markov chain method was employed to estimate the probability of significant deviation from Hardy-Weinberg equillibrium using GENEPOP (Raymond and Rousset, 1998) with parameters, dememorization=1000, batches=10 and iterations=100. The significant criteria were adjusted for the number of simultaneous tests using sequential Bonferroni technique (Lessios, 1992).

Weir and Cockeram's (1984) analogue of one of the Wright's statistics (Wright, 1978), F_{ST}, was calculated with GENETIX. To assess genetic differentiation, which is the acquisition of allele frequencies that differ among populations, in wild populations, F_{ST} values and pair wise F_{ST} values were calculated using GENEPOP (Raymond and Rousset 1998). The genotypic relationship among wild populations was estimated from Nei's standard genetic distances between all pairs of populations (Nei, 1975). The distance matrices were used to constructed UPGMA dendrogram drawn with genetic data analysis (GDA) software.

Results

4. RESULTS

The information of genetic variability and population structure information of *C. chitala* is very limited. The molecular markers suitable to detect genetic variation in this primitive fish species are also not known. Literature highlights the possible utility of microsatellite markers for genetic divergence studies of C. *chitala*.

In present study microsatellite enriched genomic library was constructed and the designed marker was used to reveal genetic variation at microsatellite loci to explore the population genetic structure of *C. chitala*.

4.1 Microsatellite enriched genomic library construction and isolation of repeat motifs

Smear of DNA inserts were obtained after digestion of genomic DNA and after primary screening with prepared repeat motifs (concatmers) amplified with SAULA primer and total 7 (out of 14 prepared concatmers) primary enriched libraries obtained for different concatmers (fig. 2). DNA fragments from primary libraries after digestion and phosphorylation cloned in the digested pUC18 vector and after transformation with DH5α cells about 5539 colonies containing both 1444 blue and 4095 white were produced for all repeat type (table. 3). Colonies after secondary screening with DIG labeled probes total 226 positive signals were detected on nylon membrane (fig. 3 e.g. of $(CA)_n$) for different repeat motifs (table. 3). Different size plasmids were isolated from all 226 (fig. 4) positive colonies and after sequencing of 190 recombinant plasmids 45 repeat regions were isolated for different repeat motifs. Sequences containing all di, tri, tetranucleotide and combination of di and tetra repeats obtained. Dinucleotde sharing maximum 55.5%, while trinucleotide 6.6%, tetra 13.3%, and mixed 24.4% obtained of the total repeat region isolated (table. 4).

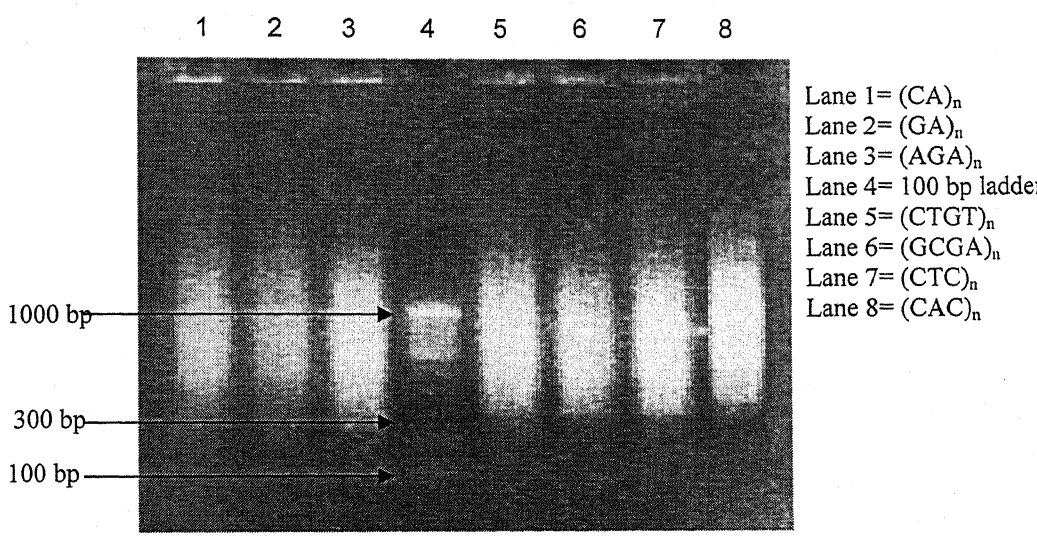


Fig. 2. Primary enriched library of *Chitala chitala* for different repeat motifs.

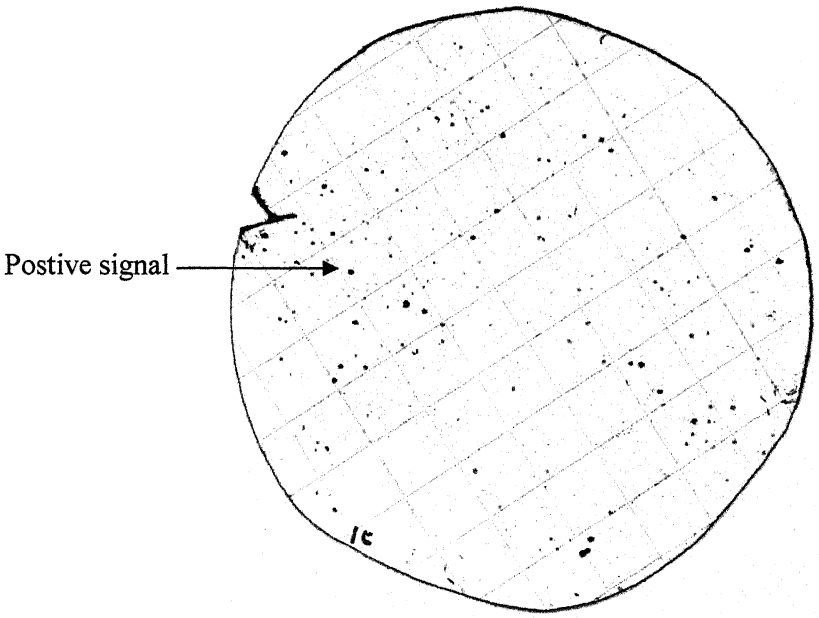


Fig. 3. Positive signals detected on nitrocellulose membrane after hybridzation e.g. for $(CA)_n$ Probe

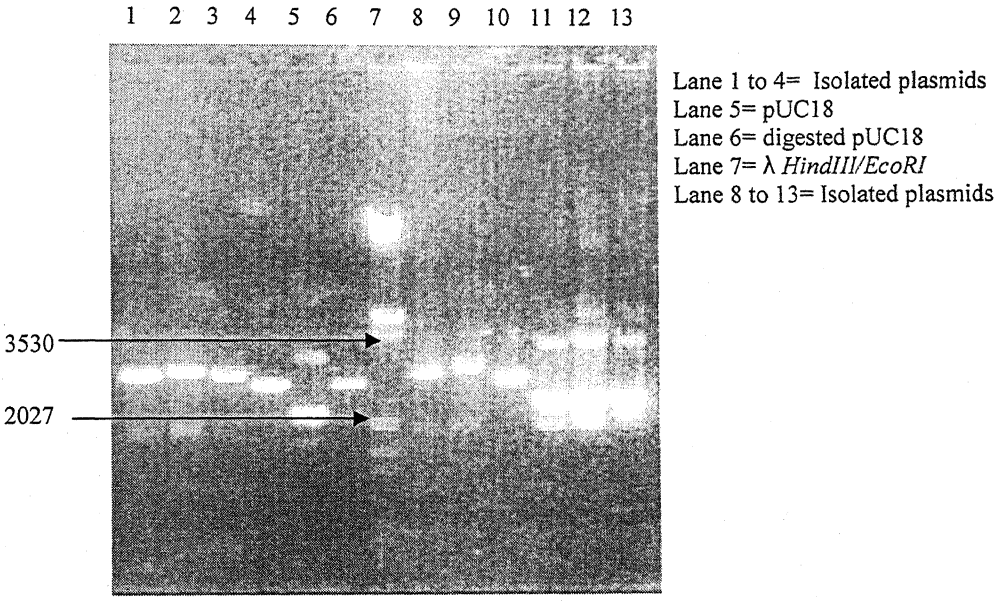


Fig. 4. Different size plasmids isolated after secondary screening of library.

Table.3. Colonies obtained from the microsatellite enriched genomic library of *Chitala chitala.*

Sl. No	Probe type	Approximate no. of colonies obtained		no. of positives after hybridzation
		White	Blue	
1.	$(CA)_{10}$	650	151	55
2.	$(GA)_{10}$	440	120	45
3.	$(CAC)_{10}$	250	59	10
4.	$(AGA)_{10}$	155	97	15
5.	$(TAT)_{10}$	110	79	09
6.	$(CTT)_{10}$	125	68	05
7.	$(CCT)_{10}$	221	103	11
8.	$(GGC)_{10}$	191	99	06
9.	(CTG)10	450	157	13
10.	$(CTC)_{10}$	251	70	17
11.	$(GACA)_8$	350	170	08
12.	$(GCAC)_8$	305	168	08
13.	$(GATA)_8$	400	60	15
14.	$(CAGA)_8$	197	43	09
	Total	**4095**	**1444**	**226**

Table.4. Microsatellite repeat types obtained from sequencing of positive clones.

Sl. No	Repeat type	Sequence	No. of clones
1.	Dinucleotide	CA	17
2.	Dinucleotide	GA	8
3.	Tetranucleotide	CAGA	1
4.	Tetranucleotide	CTGT	3
5.	Tetranucleotide	GCGA	2
6.	Trinucleotide	CTC	1
7.	Trinucleptide	CAC	2
8.	Di &Tetranucleotide	Mixed	11
		Total	**45**

4.2 Primer designing and Characterization of Microsatellite loci

Based on sequence quality and flanking length available out of 45 clones, 27 were used for the primer designing and 29 pairs of primer were designed. Microsatellite locus was given name same as the clone name. Out of 29 primer pairs tested, 8 were polymorphic, 14 were monomorhpic while 7 primers resulted in unspecific multiple product. Monomorphic microsatellite loci characterized by single band pattern (homozygote) and the primer resulted in unspecific band are not used for the genetic analysis. As such only polymorphic loci were used for the genetic analyisis of *C. chitala.* The primer sequences, clone size, repeat motif and specific annealing temperature in *C. chitala* for these primer sets are given in table. 5.

4.3 Polymorphic microsatellite loci in *Chitala chitala*

Polymorphic microsatellite loci were characterized by symmetrical two-banded pattern in heterozygote (fig. 5 to 12). Minimum size repeat was found at locus Cch6 (26 bp) while maximum size at locus Cch15 (104 bp) in size, sequences of the polymorphic loci are given in the table. 7. Alleles obtained in the range of two (Cch13) to thirteen (Cch15) and the minimum size obtained at locus Cch6 (77 bp) while Cch1 having maximum size allele (250 bp). The mean number of alleles per locus in Satluj, Bhagirathi and Brahmaputra were 4.50, 4.87 and 5.25 respectively. Characteristics of polymorphic microsatellite given in table. 6 including accession number of sequence deposited in gene bank.

4.4 Genetic variability and population structure analysis

Allele frequencies ranged from 0.0000 to 0.9333 for different locus in different population (table. 8) and loci were found to be polymorphic at both $P_{(0.95)}$ and $P_{(0.99)}$ criteria. Observed heterozygosity ranged from 0.0000

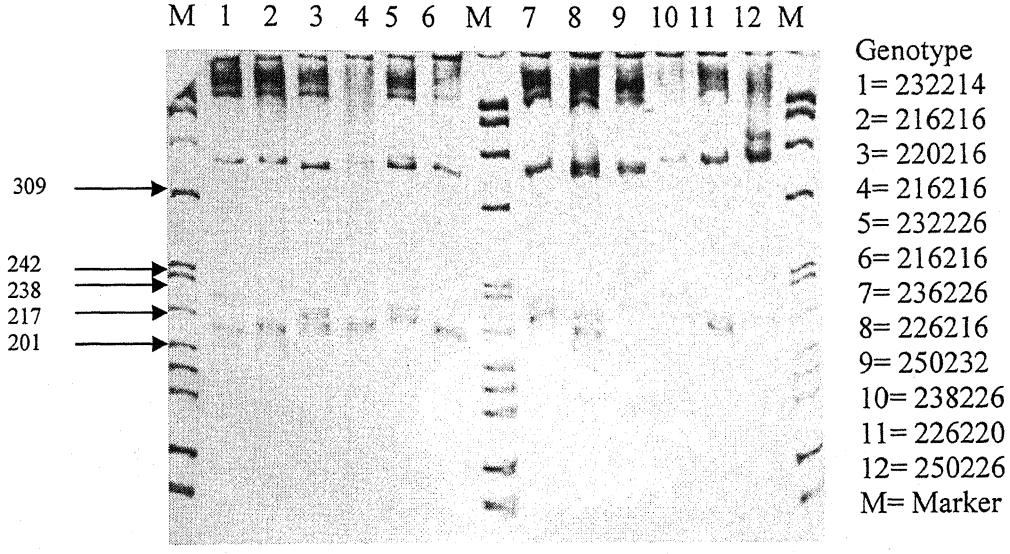


Fig. 5. Polymorphic microsatellite locus Cch1 in *Chitala chitala*.

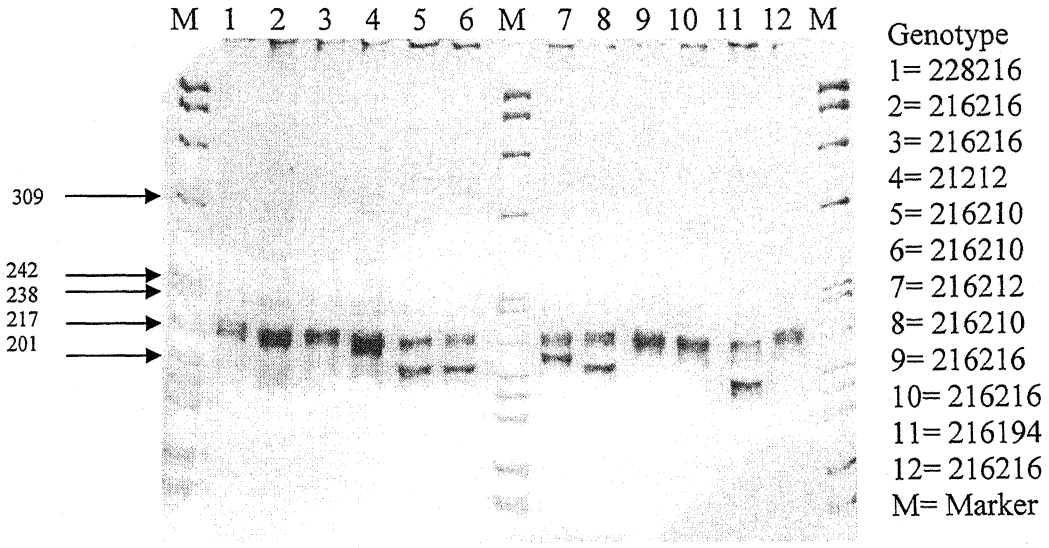


Fig. 6. Polymorphic microsatellite locus Cch2 in *Chitala chitala*.

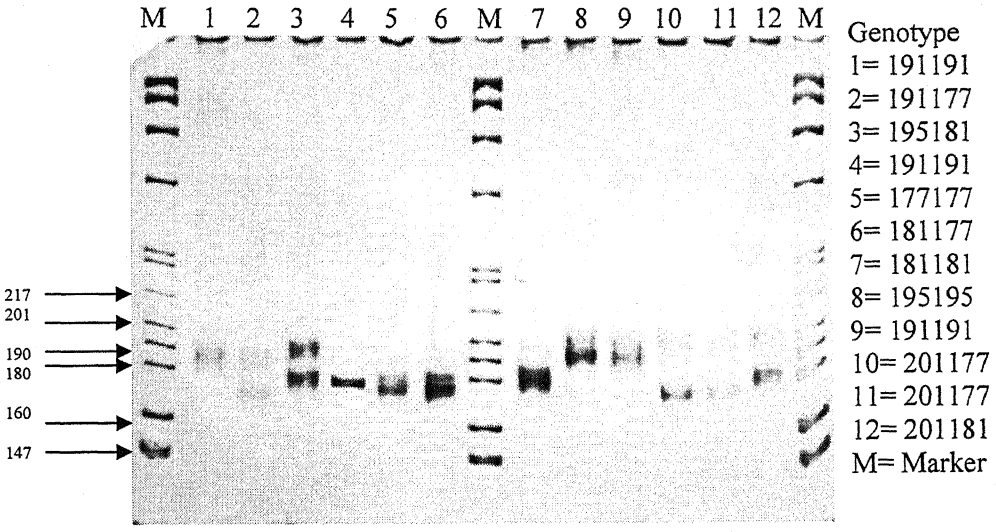


Fig. 7. Polymorphic microsatellite locus Cch4 in *Chitala chitala*.

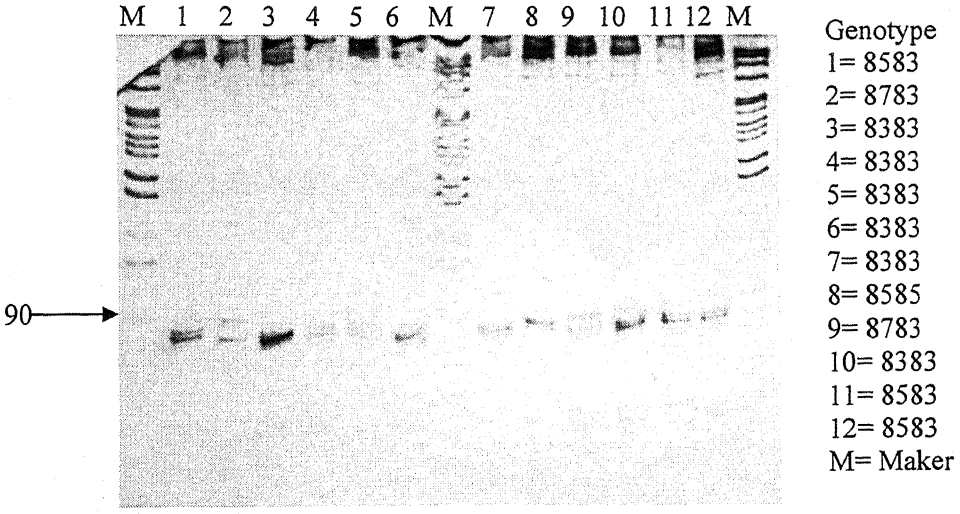


Fig. 8. Polymorphic microsatellite locus Cch6 in *Chitala chitala*.

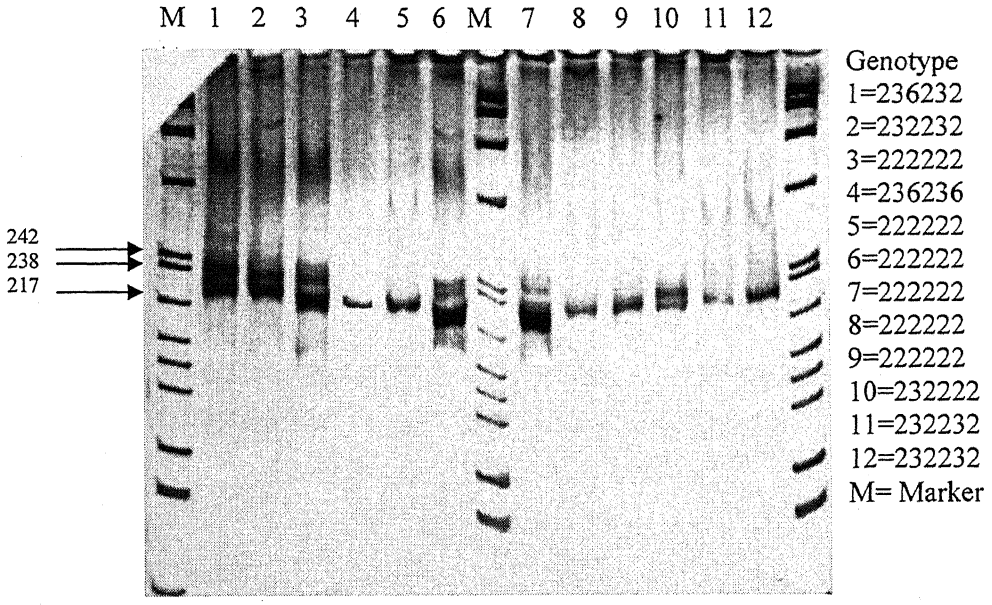


Fig. 9. Polymorphic microsatellite locus Cch9 in *Chitala chitala*.

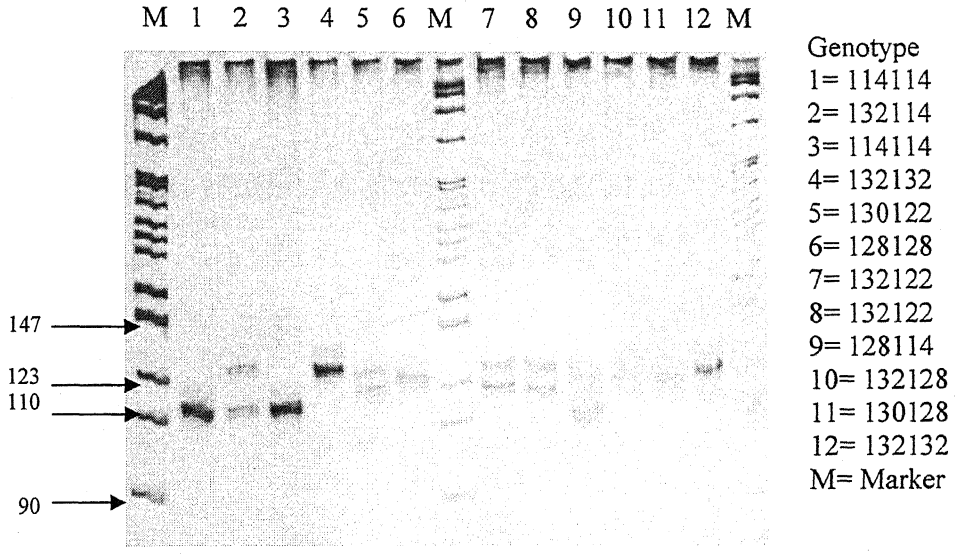


Fig. 10. Polymorphic microsatellite locus Cch10 in *Chitala chitala*.

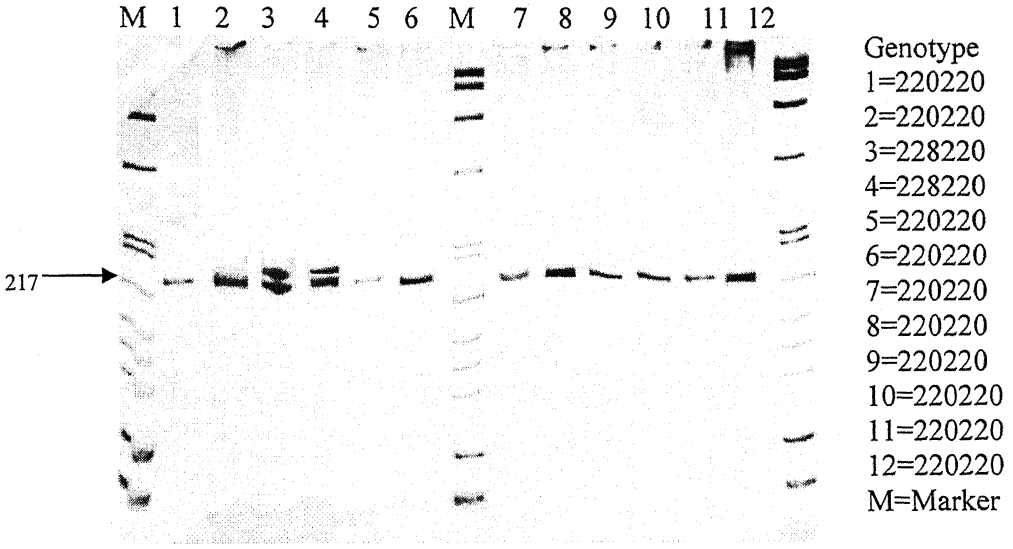


Fig. 11. Polymorphic microsatellite locus Cch13 in *Chitala chitala*.

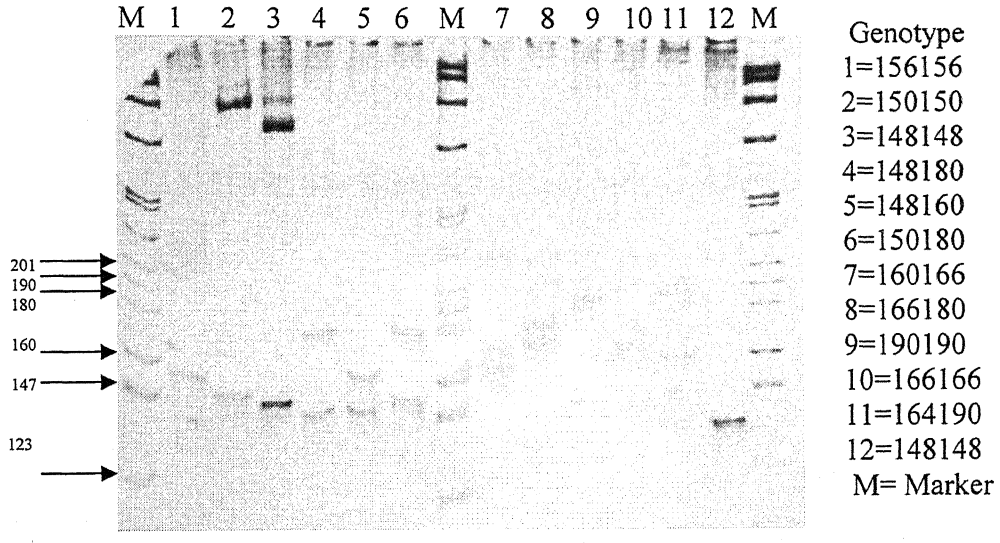


Fig. 12. Polymorphic microsatellite locus Cch15 in *Chitala chitala*.

Table. 5. Characteristics of all designed microsatellite primers in *Chitala chitala.*

Sl. No	Locus Name	Primer Sequence	Clone size	Core sequence	T_a (°C)	Band pattern
1.	Cch1	CGGAGATGAAGAGCAGCAGTA TGTGTTCCGTGTTTCCTCCTA	350bp	$(GA)_{19}(GT)_9(GA)_8$	55	Polymorphic
2	Cch2	ACCCAAGCCATGTTAAGTGGTC GGCGAGTCCACGATTTCAAG	400	$(GA)_9GTT(TG)_9$	55	Polymorphic
3	Cch3	GTGGAAATGGATGTGTAAGTGTG TTGTGTTTTAAATTTCCCCATCAT	346	$(TG)_6CAC$ $(GT)_5$	56	Monomorphic
4	Cch4	AGAGATTCCAGCCGAACCACT AGACGCAGCGCGAACTATCACAG	240	$(GA)_{29}$	60	Polymorphic
5	Cch5	TAGGATAGCAGGCACATAGCACA GGTAGCTCTTCTGGTTGTGGTGTA	368	$(CA)_9$	57	Multiple
		CAGCAAGCACCGGAACACCTA TGTGCGTGCTATCCTATGTGTA		$(CA)_{12}$	58	Multiple
6	Cch6	ATTTCCAGCTTCTAACCGCACACC AGTTGGGGATGCCGCTGTC	300	$(CA)_{13}$	57	Polymorphic
7	Cch7	TTCTGGAGAATGCAGTGGTCTGT TCATGGTCATGTTAAATGCAATTT	212	$(CT)_6(CA)_{12}$	55	Muttiple
8	Cch8	GAGAGCCGTGATGACAGAGGA GACCCCACCAGGCTCGTTAG	489	$(GCGA)_4$	61	Monomorphic
9	Cch9	TGGTGTTGGAGTGTGAGTGCTTAG ATATGCAGTGGCACGACAGGT	289	$(TC)_{12}$ $(AC)_{14}$	60	Polymorphic
10	Cch10	TCGTTATTTCTGACATTCAAGTGC TACAAGCTCCATGCACAATTACAA	300	$(GT)_{21}$	53	Polymorphic
11	Cch12	GTTTGCGAGACATGGAGTGTGG CATTTGGACATATGACTTCCTGTATT	260	$(AC)_5G(CA)_5$	55	Multiple
12	Cch13	AAGGGTACTGATGAGTGAATGAGC TCATAACAGGCTGTTTATTGTCCA	300	$(CT)_{14}$	55	Polymorphic
13	Cch14	CTGGCCCTGGCACTGTAGCA CCTCAGCCATGGCGTCGTG	154	$(GA)_7GC(GA)_9$	60	Monomorphic
14	Cch15	AACACTGAGCGAAAAGCAACA GATAAACGGGTGAGAGCAAGTG	214	$(GA)_5[CA(GA)_3]4[CA$ $(GA)_2]3CA(GACA)_{11}$	55	Polymorphic
15	Cch16	GTTTCAAGTCACCGGTGTCTGTAA TTCCAGAGGAGATGCAGACATTC	415	$(GT)_7CCC(TG)_8CCC$ $(TG)_{15}C(TG)_{18}$	57	Monomorphic
16	Cch17	TGTTACATGTCCTGGTTTCCTCTC GTTTGTCAGTGGTGCACGTCAG	333	$(TG)_8C(GT)_7$	57	Monomorphic
17	Cch18	TGTAGGAGCCGGAGTGGGAGAA CTGCAGCAGTAGGCCTGTGAGTG	340	$(CA)_{11}$	60	Monomorphic
18	Cch19	GATAAGGCTGCGCAGTCGTCT GGCCTCTCAGGGTGCTCTTACAT	373	$(CA)_{13}$---$(GA)_7$	57	Monomorphic
19	Cch20	GGAGGGATGCTGTGCACTATAAAG CCGTTGGGTCTGTCTGTATATCTG	300	$(GA)_{12}$	55	Monomorphic
20	Cch21	CATCTTCTATACCGCTTCATCGTG TGTGTCCAAATGTCCCATAGC	181	$(CA)_7$	60	Multiple
21	Cch25	CCAAGATTATAACGGGTGAGA ATTATTAGCATGGTCTTCAGTG	172	$(CTGT)_9$	57	Monomorphic
22	Cch26	CAACACTGAGGCAAAAGCAACA GATAAACGGGTGAGAGCAAGTG	209	$(CAGA)_{11}$	55	Monomorphic
23	Cch29	AAGGCAGGCTCTTCAACAACTGATT GGGGTGGAAGAGCCAAGGTG	220	$(CAGA)_{10}$	55	Monomorphic
24	Cch33	GACAGGCAGCTCTTCAACACT CCCGGGGATTCGATAAAC	240	$(CAGA)_{11}$	55	Monomorphic
25	Cch36	TCCAGAGGATTTGAAGTCC GGAAACAGCTATGACATGATTACG	238	$(GT)_6$	57	Multiple
26	Cch38	CAACATCGGTGAAGCTACAGAGG AAAAATGTTGTGCTGCTATCCGT	247	$(CA)_{13}$	57	Monomorphic
		CAACATCGGTGAGCTACAGA GAAACAGCTATGACATGATTACGA			55	Monomorphic
27	Cch39	TCAAGCATGCGTGTTAGTGTATGG TGGGGAGCGGGGGTTGTT	402	$(GT)_9$	55	Multiple

Table. 6. Charateristics of polymorphic microsatellite markers.

Sl. No	Locus Name	Accession no.	River/ Population	NA*	Size Range	Total allele
1	Cch1	DQ525389	Satluj	4	216-232	7
			Bhagirathi	6	220-250	
			Brahmaputra	6	216-238	
2	Cch2	DQ525390	Satluj	4	202-228	5
			Bhagirathi	5	194-228	
			Brahmaputra	5	194-228	
3	Cch4	DQ525391	Satluj	7	117-205	7
			Bhagirathi	7	177-205	
			Brahmaputra	7	177-205	
4	Cch6	DQ525392	Satluj	3	83-87	4
			Bhagirathi	3	83-87	
			Brahmaputra	4	77-87	
5	Cch9	DQ525393	Satluj	3	222-236	3
			Bhagirathi	3	222-236	
			Brahmaputra	3	222-236	
6	Cch10	DQ525394	Satluj	5	114-132	6
			Bhagirathi	6	114-132	
			Brahmaputra	6	114-132	
7	Cch13	DQ525395	Satluj	2	220-228	2
			Bhagirathi	2	220-228	
			Brahmaputra	2	220-228	
8	Cch15	DQ525396	Satluj	8	130-198	13
			Bhagirathi	7	130-146	
			Brahmaputra	10	138-184	
		Mean	Satluj	4.50		
			Bhagirathi	4.87		
			Brahmaputra	5.25		

***NA = Number of alleles**

Table.7. DNA sequences of the polymorphic microsatellite loci.

Cch1

CGGAGATGAAGAGCAGCAGTATACATAACAATGTTAAAGCGTAGGGAAATACGCAGATACAGTAGT
GTGGGTCAGGGCGTTGGTGCCTGGAAAGAAAGAAAGAAAGAGAAAGAGAGAGAGAGAGAGAGAG
AGAGAGAGAGAGAGAGAGAGTGTGTGTGTGTGTGTGTGAGAGAGAGAGAGAGAAAACAGCAGTTT
GTAGGAGGAAACACGGAACACACTGGGTCCGTGTTGTAATGAGGCTGATTCTGTCCTGGATCGCAAA
TGTGGGATAAAACAACTAATCTGTGACTTTGAAATGGTGCGCATTTTGTCATGCGTCCTCCAGCGGG
ACGAGCTTTTACTTTGCGCGC

Cch2

AGGATATACAATGTGCTCACCGCACCTTTAGTTATTATCCTGTTTGTTGGTAAGAGCAGGTTTTTCTT
TTTTTAGTGGGATAAAAACTTTCACCCCAGACCCAAGCCATGTTAAGTGGTCCAAGTAAACTGTACT
GCAATTTAGCAACTTTAAAGAGAGAAAGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
AGAGTTTGTGTGTGTGTGTGTGTGTTGCACAGAGGAACACTGTTGAATGTTAATTTGCTAGGCTTGTT
CTGATTAATTTTTTGTTGCGCTTGAAATCGTGGACTCGCCACTGAATCACGTAATGCGCAAAGGATTT
ATTAGGAATAACGTGAGCGCTGCTTTCGTGTTGTGCCAACTGCAACGCCAGTTCCAAGGGTCGAC

Cch4

TTCCTCCTAGCAGTGCTATGTGAGAGATTCCAGCCGCACCACTGGATTTCAGGCTGAGATTTATCCAC
TCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGCG
CTTCGGCCTGACAGAGCCCCCTCTCCAATTTGTCCATTTATCTATGGCCTGTGATAGTTCGCGCTGCG
TCTGCACAAATTTTGCGTTGTTGGCAAGCAAACAGGAGAA

Cch6

CCCCAGTGTAAGCAGCTTGGAAGGTTTCATAGCCTCGATTTCCAGCTTCTAACCGCACACCGCACAC
ACACACACACACACACACACACACGGAGGGTGTGGGACAGCGGCATCCCCAACTGCGACGAATTTTAA
GATGTCGTTATATTTTCGAATGAACGTTTTTTTCTTTTAGACTCTGCTTTCTCTTTGTAATCCGCCCCA
GTGTTTCAGCAGTGCCAAAAAAGAGGGGAAAAAACACTTGTTAACGTACTTTATTCGTTTATTTCCCC
GCACAGAAAGCTCATTTTGCTTTATTGTATG

Cch9

AAATGGTGTTTCATTTGTAGAGCAGGGGAGGAATGACCTGCACTGTTAAGTACATGGTGTTGGAGTG
TGAGTGCTTAGCAGAGAACAGCACACAGACGTGAGGGCCATCTCACGGGTGACACACAGGAGAGGC
ATCTCTCTCTCTCTCTCTCTCTCACACACACACACACACACACACACACACACGGCTCTGAGAGCTGA
GAGATGTTTCCCATCGCTGACAGGCGGCAAGACCTGCATTTCTGGCCTTTGACAGCACCAGTATAAC
CTGTCGTGCCACTGCATATCC

Cch10

TTTTTCCTTACCCTCTATATCTAAAAATATGCTGGATGCTAGTGGTATCGGGTTGGAAATTGTGTATA
ATAAAAGCTTTTAAGTCATTAAGCTAATTGTATGCATACTATTCCACTCTGTCGTTATTTCTGACATTC
AAGTGCATTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGTGCATGTGTGTGT
TTGGCCTGCCTTGGTTTTTGTAATTGTGCATGGAGCTTGTAGAGCATCGTCCCTTTGTTTCACGGAGA
AAAGGAAACGCGGTGAAACTTTAAATC

Cch13

TAAGGGTACTGATGAGTGAATGAGCGTGGTTAACCACGTGCATTTCTCTCTCTCTCTCTCTCTCTC
TCTCTCCTCATGTTGCTGAAGCCCCGCCTCCATCGAGCCTGGTGTCAGCCACAGTGACCAGGTCATTA
TGAAGGACTGAGCATTAACCGCTGGTACCAGAGCCAAGCATGACTGAAAAAATACAGCTCGTCAGA
TGGACAATAAACAGCCTGTTATGAACACCATCGCCTAACAGGACATCAAATTTCCCAGTGTGGATTT
TAATAGCAACGGAGGTCTGTAACCACATACG

Cch15

TGACAGGCAGCTCTTCAACACTGAGCGAAAAGCAACACACCCAAAATATTATTAGCATGGTCTTCAG
TGACAGAGAGAGAGACAGAGAGACAGAGAGACAGAGAGACAGAGAGACAGAGACAGAGACAGAG
ACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAAGCACTTGCTCTCACCCG
TTTATCGATCCCCGGGTA

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*Underlined area showing the repeat region in the sequence

(Bhagirathi and Brahmaputra samples, locus Cch13) to 0.8571 (Bhagirathi samples, locus Cch10) while the expected heterozygosity ranged from 0.1244 (Brahmaputra samples, locus Cch13) to 0.8571 (Bhagirathi samples, locus Cch15). Heterozygosity value (H_{obs} and H_{exp}), probability of conformance to expected Hardy-Weinberg proportion per locus per population, F_{IS} (inbreeding coefficient) and P_{score} (heterozygote deficient) are given in table. 9.

Genotype proportion at eight polymorphic loci was tested for departure from Hardy-Weinberg equilibrium expectation and significant deviation was evident in Bhagirathi and Brahmaputra samples at locus Cch9 after sequential Bonferroni correction ($P<0.002$) were applied to the critical probability level ($P = 0.05$). Observed deviation evident as difference in the H_{obs} and H_{exp} is high on this locus. More powerful score test (Raymand and Rousset, 1995) that assesses against specific alternate hypothesis of heterozygote deficiency or excess also confirmed the above inference. The observed deviation from the Hardy-Weinberg the expectation was associated with deficiency of heterozygote in Bhagirathi and Brahmaputra samples Cch9 locus as F_{IS} value on this locus is significantly positive (table. 9). Negative values of F_{IS} at some loci indicate excess of observed heterozygote then expected.

Probability value of genotypic disequilibrium ranged from 0.075952 to 1.000000 which is greater than significant value ($P>0.05$) for the pair of loci in any samples and across all the samples (table. 10). This indicated that genotypes are independent and not linked at these microsatellite loci for *C. chitala*.

Genotype proportions from multiple data sets (collection in different years) in rivers Satluj and Bhagirathi were tested for homogeneity. The test did not indicate any significant divergence between these sets within a river ($P>0.05$). The genotype data sets within a river were pooled and total three combined genotype data sets (Satluj, Bhagirathi and Brahmaputra,) were available, for further investigation of genetic variation and differentiation.

Table. 8. Allele size and frequency at ploymorphic locus

Locus	Allele Size	Satluj	Bhagirathi	Brahmaputra
Cch1	250	0.0000	0.1429	0.0000
	238	0.0000	0.1429	0.1000
	236	0.0000	0.0714	0.0667
	232	0.0333	0.1429	0.1667
	226	0.0667	0.1429	0.2667
	220	0.7333	0.3571	0.3667
	216	0.1667	0.0000	0.0333
Cch2	228	0.1000	0.2000	0.0333
	216	0.6333	0.5667	0.6333
	210	0.1667	0.1000	0.1000
	212	0.1000	0.1000	0.2333
	194	0.0000	0.0333	0.0000
Cch4	205	0.0714	0.0385	0.0357
	201	0.0357	0.0769	0.0714
	195	0.0357	0.1538	0.1786
	191	0.4643	0.2692	0.0714
	185	0.1429	0.0385	0.1071
	181	0.1429	0.2308	0.3929
	177	0.1071	0.1923	0.1429
Cch6	87	0.2000	0.3846	0.1000
	85	0.1000	0.1923	0.4000
	83	0.7000	0.4231	0.4333
	77	0.0000	0.0000	0.0667
Cch9	236	0.2333	0.1000	0.3000
	232	0.1333	0.5333	0.4000
	222	0.6333	0.3667	0.3000
Cch10	132	0.2857	0.1071	0.1000
	130	0.2857	0.2143	0.2000
	128	0.1071	0.2143	0.2667
	122	0.0000	0.0714	0.3333
	118	0.0357	0.0714	0.0333
	114	0.2857	0.3214	0.0667
Cch13	228	0.1071	0.0769	0.0667
	220	0.8929	0.9231	0.9333
Cch15	198	0.0385	0.0000	0.0000
	192	0.0000	0.1429	0.0357
	184	0.0000	0.0000	0.0714
	180	0.0000	0.0714	0.0357
	178	0.1154	0.1429	0.1071
	176	0.0385	0.0714	0.1071
	168	0.0000	0.2143	0.2857
	164	0.1154	0.0000	0.0714
	154	0.1538	0.2857	0.0000
	152	0.4231	0.0714	0.0000
	142	0.0769	0.0000	0.0714
	134	0.0000	0.0000	0.1429
	130	0.0385	0.0000	0.0714

Table. 9. Parameters of genetic variation for the eight microsatellite loci in *Chitala chitala* from three riverine systems.

Locus	Population	Hexp	Hobs	PHW	Pscore	F_{IS}
Cch1	Satluj	0.4289	0.4000	0.7353	0.3666	0.1016
	Bhagirathi	0.7857	0.6429	0.2293	0.0236	0.2174
	Brahmaputra	0.7511	0.7333	0.1798	0.3192	0.0581
Cch2	Satluj	0.5511	0.4667	0.1553	0.0950	0.1867
	Bhagirathi	0.6178	0.3333	0.0033	0.0050	0.4872
	Brahmaputra	0.5333	0.6000	0.6585	0.7929	-0.0909
Cch4	Satluj	0.7245	0.5714	0.0784	0.0785	0.2464
	Bhagirathi	0.8047	0.4615	0.0042	0.0047	0.4586
	Brahmaputra	0.7704	0.5000	0.0310	0.0024	0.3831
Cch6	Satluj	0.4600	0.4667	0.7760	0.5247	0.0200
	Bhagirathi	0.6361	0.5385	0.2298	0.1240	0.1923
	Brahmaputra	0.6378	0.4667	0.0422	0.1482	0.3000
Cch9	Satluj	0.5267	0.3333	0.0081	0.0042	0.3966
	Bhagirathi	0.5711	0.2000	0.0002*	0.0314	0.6693
	Brahmaputra	0.6600	0.1333	0.0001*	0.0000	0.8102
Cch10	Satluj	0.7423	0.5000	0.0148	0.0389	0.3592
	Bhagirathi	0.7832	0.8571	0.3128	0.7930	-0.0576
	Brahmaputra	0.7622	0.8000	0.6269	0.5646	-0.0151
Cch13	Satluj	0.1913	0.2143	1.0000	1.0000	-0.0833
	Bhagirathi	0.1420	0.0000	0.0413	0.0413	1.0000
	Brahmaputra	0.1244	0.0000	0.0331	0.0331	1.0000
Cch15	Satluj	0.7604	0.6154	0.3547	0.0424	0.2289
	Bhagirathi	0.8163	0.7143	0.0734	0.0866	0.2000
	Brahmaputra	0.8520	0.5714	0.0043	0.0000	0.3620
Mean overall loci	Satluj	0.5481	0.4460			
	Bhagirathi	0.6446	0.4685			
	Brahmaputra	0.6364	0.4756			

H_{obs} - Observed heterozygosity.
H_{exp} - Expected heterozygosity.
F_{IS} - Inbreeding coefficient.
P_{HW} - Probability value of significant deviation from Hardy-Weinberg equilibrium.
P_{score} - Probability value of significant heterozygosity deficiency.
* Significant after sequential Bonferroni correction ($P<0.002$).

Table. 10. Linkage disequilibrium P-value for *Chitala chitala* microsatellites.

Population	Locus Pair	P-Value	S.E.
Satluj	Cch1 Cch2	0.670144	0.004476
Satluj	Cch1 Cch4	0.894018	0.005067
Satluj	Cch2 Cch4	0.871836	0.004452
Satluj	Cch1 Cch6	0.509154	0.004648
Satluj	Cch2 Cch6	0.987080	0.000646
Satluj	Cch4 Cch6	0.723964	0.005560
Satluj	Cch1 Cch9	0.942876	0.001737
Satluj	Cch2 Cch9	0.811420	0.002937
Satluj	Cch4 Cch9	0.973078	0.001645
Satluj	Cch6 Cch9	0.271120	0.003347
Satluj	Cch1 Cch10	0.075952	0.002920
Satluj	Cch2 Cch10	0.914262	0.002658
Satluj	Cch4 Cch10	1.000000	0.000000
Satluj	Cch6 Cch10	0.127396	0.003263
Satluj	Cch9 Cch10	0.234466	0.004233
Satluj	Cch1 Cch13	1.000000	0.000000
Satluj	Cch2 Cch13	0.790836	0.001247
Satluj	Cch4 Cch13	0.958064	0.000986
Satluj	Cch6 Cch13	0.171192	0.001340
Satluj	Cch9 Cch13	1.000000	0.000000
Satluj	Cch10 Cch13	1.000000	0.000000
Satluj	Cch1 Cch15	1.000000	0.000000
Satluj	Cch2 Cch15	1.000000	0.000000
Satluj	Cch4 Cch15	1.000000	0.000000
Satluj	Cch6 Cch15	0.676342	0.007208
Satluj	Cch9 Cch15	0.790834	0.004800
Satluj	Cch10 Cch15	1.000000	0.000000
Satluj	Cch13 Cch15	0.424738	0.004463
Bhagirathi	Cch1 Cch2	0.556818	0.011904
Bhagirathi	Cch1 Cch4	1.000000	0.000000
Bhagirathi	Cch2 Cch4	1.000000	0.000000
Bhagirathi	Cch1 Cch6	1.000000	0.000000
Bhagirathi	Cch2 Cch6	0.306760	0.006961
Bhagirathi	Cch4 Cch6	0.033160	0.002507
Bhagirathi	Cch1 Cch9	0.784614	0.005749
Bhagirathi	Cch2 Cch9	0.082402	0.002511
Bhagirathi	Cch4 Cch9	1.000000	0.000000
Bhagirathi	Cch6 Cch9	0.946498	0.001497
Bhagirathi	Cch1 Cch10	1.000000	0.000000
Bhagirathi	Cch2 Cch10	0.361430	0.009277
Bhagirathi	Cch4 Cch10	1.000000	0.000000

Population	Locus Pair	P-Value	S.E.
Bhagirathi	Cch6 Cch10	0.520864	0.008481
Bhagirathi	Cch9 Cch10	0.391998	0.007184
Bhagirathi	Cch1 Cch13	0.774776	0.003586
Bhagirathi	Cch2 Cch13	0.154608	0.001644
Bhagirathi	Cch4 Cch13	0.814282	0.003190
Bhagirathi	Cch6 Cch13	1.000000	0.000000
Bhagirathi	Cch9 Cch13	1.000000	0.000000
Bhagirathi	Cch10 Cch13	0.532266	0.004423
Bhagirathi	Cch1 Cch15	1.000000	0.000000
Bhagirathi	Cch2 Cch15	0.144636	0.006196
Bhagirathi	Cch4 Cch15	1.000000	0.000000
Bhagirathi	Cch6 Cch15	1.000000	0.000000
Bhagirathi	Cch9 Cch15	1.000000	0.000000
Bhagirathi	Cch10 Cch15	1.000000	0.000000
Bhagirathi	Cch13 Cch15	0.713284	0.002350
Brahmaputra	Cch1 Cch2	0.780544	0.006782
Brahmaputra	Cch1 Cch4	1.000000	0.000000
Brahmaputra	Cch2 Cch4	0.428860	0.007754
Brahmaputra	Cch1 Cch6	0.165150	0.005968
Brahmaputra	Cch2 Cch6	0.379132	0.004572
Brahmaputra	Cch4 Cch6	1.000000	0.000000
Brahmaputra	Cch1 Cch9	0.949170	0.002603
Brahmaputra	Cch2 Cch9	0.673658	0.004637
Brahmaputra	Cch4 Cch9	0.833206	0.007450
Brahmaputra	Cch6 Cch9	0.395780	0.004281
Brahmaputra	Cch1 Cch10	1.000000	0.000000
Brahmaputra	Cch2 Cch10	0.896164	0.004045
Brahmaputra	Cch4 Cch10	0.322556	0.016757
Brahmaputra	Cch6 Cch10	1.000000	0.000000
Brahmaputra	Cch9 Cch10	0.834658	0.005126
Brahmaputra	Cch1 Cch13	0.396858	0.003676
Brahmaputra	Cch2 Cch13	1.000000	0.000000
Brahmaputra	Cch4 Cch13	0.712422	0.005625
Brahmaputra	Cch6 Cch13	0.268648	0.001957
Brahmaputra	Cch9 Cch13	0.131346	0.001771
Brahmaputra	Cch10 Cch13	0.801728	0.003240
Brahmaputra	Cch1 Cch15	1.000000	0.000000
Brahmaputra	Cch2 Cch15	0.414498	0.009061
Brahmaputra	Cch4 Cch15	1.000000	0.000000
Brahmaputra	Cch6 Cch15	0.286534	0.009112
Brahmaputra	Cch9 Cch15	1.000000	0.000000
Brahmaputra	Cch10 Cch15	1.000000	0.000000
Brahmaputra	Cch13 Cch15	0.714688	0.004057

Spatial homogeneity in genotype distribution at each microsatellite loci was tested over all populations. When considered for each locus, Bhagirathi and Brahmaputra samples were having significant heterogeneity value to Satluj samples at Cch1 locus after sequential Bonferroni correction ($P<0.002$). Brahmaputra samples had significant differences with Satluj samples at Cch10 and highly significant value was observed between Brahmaputra and Satluj at locus Cch15 (table. 11). Probability values for each population pair across all loci based on Fisher's exact test indicates that except Bhagirathi and Brahmaputra population pair significant heterogeneity is found with other population pairs after sequential Bonferroni correction ($P<0.002$). Pairwise comparison of F_{ST} values in between Satluj & Bhagirathi, Satluj & Brahmaputra and Bhagirathi & Brahmaputra were 0.048753, 0.089690 and 0.009773 respectively (table. 12) and F_{ST} estimates over all loci in all population were found to 0.052405. UPGMA dendrogram constructed (fig. 13) indicated the genetic similarity and divergence between the pair of populations.

Table.11. Pairwise comparison of allele homogeneity allele homogeneity in *Chitala chitala* microsatellite loci.

Locus	Population pair	P-Value
Cch1	Bhagirathi & Satluj	0.00007*
	Brahmaputra & Satluj	0.00182*
	Brahmaputra & Bhagirathi	0.30760
Cch2	Bhagirathi & Satluj	0.66969
	Brahmaputra & Satluj	0.41092
	Brahmaputra & Bhagirathi	0.14926
Cch4	Bhagirathi & Satluj	0.42681
	Brahmaputra & Satluj	0.02459
	Brahmaputra & Bhagirathi	0.58549
Cch6	Bhagirathi & Satluj	0.13539
	Brahmaputra & Satluj	0.01309
	Brahmaputra & Bhagirathi	0.02614
Cch9	Bhagirathi & Satluj	0.00351
	Brahmaputra & Satluj	0.02489
	Brahmaputra & Bhagirathi	0.17890
Cch10	Bhagirathi & Satluj	0.34630
	Brahmaputra & Satluj	0.00068*
	Brahmaputra & Bhagirathi	0.07124
Cch13	Bhagirathi & Satluj	1.00000
	Brahmaputra & Satluj	0.66465
	Brahmaputra & Bhagirathi	1.00000
Cch15	Bhagirathi & Satluj	0.00922
	Brahmaputra & Satluj	0.00000*
	Brahmaputra & Bhagirathi	0.04520

* Significant after sequential Bonferroini correction (P<0.002).

Table.12. Pairwise F_{ST} and Probability in *Chitala chitala* microsatellites. (F_{ST} above diagonal, probability below diagonal).

Populations Pair	Satluj	Bhagirathi	Brahmaputra
Satluj	*******	0.048753	0.089690
Bhagirathi	0.000041*	*******	0.009773
Brahmaputra	0.000000*	0.021135	*******

* Significant after sequential Bonferroini correction (P<0.002).

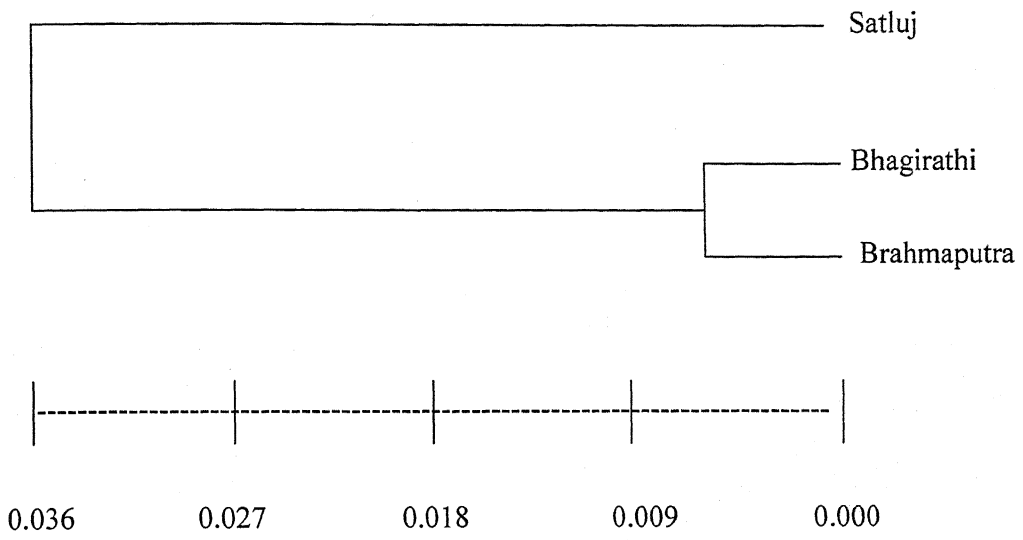

Fig. 13. UPGMA dendrogram based on microsatellite loci in natural population of *Chitala chitala* in India.

Discussion

5. DISCUSSION

Microsatellites consists of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs (Tautz, 1989; Litt and Luty, 1989). Abundant in all species studied to date, microsatellites have been estimated to occur as often as once every 10 kb in fishes (Wright, 1993). Microsatellites are inherited in a Mendelian fashion as codominant marker, this is another strength of microsatellite markers in addition to their abundance, even genomic distribution, small locus size, and high polymorphism. Microsatellite primers from one species can be used for amplification of polymorphic microsatellite loci from species of same family or cross species. High polymorphism observed in a species does not guarantee that similar polymorphism will be found in related species (Rubinsztein *et. al.*, 1995; Morin *et al.*, 1998). This becomes a major hindrance to study genetic variation and hence required development of suitable microsatellite markers for study of genetic variations. The main focus of the study is constructing microsatellite enriched genomic library of *C. chitala* to isolate and characterize microsatellite markers. The study also aims to explore pattern of genetic variation in *C. chitala* across its natural range of distribution. Three riverine populations (Satluj, Brahmaputra and Bhagirathi) were chosen to cover geographically distant populations of *C. chitala.*

5.1 Micosatellite enriched genomic library construction, isolation and characterization of polymorphic loci.

In this study from microsatellite enriched genomic library constructed for different types of repeats i.e. di, tri and tetra. 190 clones were sequenced out of which 45 (23.6%) were found to have microsatellite sequences. This indicated successful selective enrichment of library for microsatellite as against 0.04% to 12% of positive clones containing microsatellites obtained in the

traditional method (Zane *et. al.*, 2002). Out of these 55.5% of dinucleotide repeat obtained from the library confirmed the presence of their higher frequency and as compared to tri and tetra microsatellite higher percentage (37.7%) isolation of CA/GT dinucleotide repeat indicated that these are found more in number comparison of other types in the fish genome (Tautz *et. al.*, 1986).

Out of 22 primer pairs amplified 36.6% (8 loci) were polymorphic loci while 63.6% (14 loci) monomorhpic loci isolated from the partially enriched genomic library. Microsatellite loci found polymorphic at both $P_{(0.95)}$ and $P_{(0.99)}$ criteria and the number of variable size allele obtained (2 to 13) indicated highly variable property of microsatellites markers as mutation rate found at microsaellite loci very high (Bryan and Mart, 2001).

5.2 Genetic variability and population structure analysis

5.2.1 Genetic variability parameters in *C. chitala*

The study provided information on the genetic variation parameters at 8 polymorphic microsatellite loci to explore variation and the population structure of *C. chitala*. Allele frequencies ranged from 0.0000 to 0.9333 for different loci in different set of samples studied. Observed heterozygosity ranged from 0.0000 to 0.857 while the expected heterozygosity ranged from 0.1244 to 0.8571. No linkage disequilibrium was observed between all the eight polymorphic in any of the population studied and these all loci can be used for genetic variability studies ($P>0.05$). Genotype proportion at eight polymorphic loci was tested for departure from Hardy-Weinberg equilibrium expectation and significant deviation was evident in Bhagirathi and Brahmaputra samples at locus Cch9 as F_{IS} value on this locus is highly positive (table. 9). The observed deviation from Hardy-Weinberg equilibrium was associated with deficiency of heterozygote and the null alleles may be cause of concern that can give rise to deficiency of

heterozygotes in the computation for microsatellite data (Paetkau and Strobeck, 1995; Ishibashi *et. al.*, 1996; Jones *et. al.*, 2001).

5.2.2 Genetic divergence studies in *C. chitala*

Fine scale analysis of *C. chitala* samples with microsatellite markers from different collection localities revealed existence of population subdivision. The combined F_{ST} value 0.052405 for all loci in all three populations indicated genetic divergence present between three rivers. Satluj samples are significantly divergent from that of Brahmaputra and Bhagirathi (F_{ST} = 0.089690 and 0.048753 respectively) while Brahmaputra and Bhagirathi (F_{ST} = 0.009773) showed lower level of divergence. The genetic variation is the outcome of several interactive evolutionary forces that act on the natural population (Ryman, 2002). Most important are migration, random genetic drift and mutation. In the present scenario the rivers of Indus, Ganges and Brahmaputra are not interlinked. There is clear evidence of the existence of Indo-Brahma river that used to flow from present Assam to northwest to fall in present Arabian sea after Gondwanaland dashed against Eurasia plate (Daniels, 2001). Tectonic movements caused changes in river courses and Ganges developed in front of Himalayas and Indus, Brahmaputra and Ganges become separate rivers out of Indo-Brahma river. It is quite likely the ancestral stock could have been the same or the genepool with gene flow and change in the course of rivers fragmented the populations. UPGMA dendrogram indicated that Bhagirathi and Bhramputra are genetically related while Satluj is more divergent from both the population samples (fig.13).

The results revealed the evidence of sub-structuring in *C. chitala* population in three river studies. Combined F_{ST} value 0.052405 and significant genetic divergence evident between Satluj and other population also confirm the statement. The less genetic differentiation between Bhagirathi & Brahmaputra may due to sharing of common gene pool. Though it may not be

conclusive evidence and studies of more number of samples are required for ascertaining the divergence level.

In conclusion, the microsatellite markers developed from the partially enriched genomic library of *C. chitala* provided useful information on genetic variation present between and within the natural populations and its population structure. The results obtained will be useful for future researcher in further exploitation on population structure, evolutionary relationships, conservation of natural resources of *C. chitala* and also for genetic improvement programmes.

Summary

6. SUMMARY

Chitala chitala, commonly known as Feather-back or "Old World Knife Fish", belongs to the order Osteoglossiformes, family Notopteridae (Nelson, 1994). *C. chitala* is a warm water fish native of Indian subcontinent including India, Bangladesh, Myanmar, Nepal and Pakistan (Froese and Pauly, 2003). It has very high commercial value as food fish as well as for the ornamental trade. The sharp decline in abundance of *C. chitala* and its endangered status is indeed a serious concern. For the significance attached to the species, effective conservation and propagation assisted rehabilitation strategies are needed to be planned and the information of genetic variation found in *C. chitala* will be very useful for effective management. The information of genetic variability and population structure information of *C. chitala* is very limited. The molecular markers suitable to detect genetic variation in this primitive fish species are also not known. Literature highlights the potential utility of microsatellite markers for genetic divergence studies of *C. chitala* (Mandal, 2005).

This study was undertaken to identify the suitable polymorphic genetic markers and to determine the genetic variation within and between the populations of *C. chitala* for population structure analysis.

6.1 Construction of microsatellite enriched genomic library and characterization of polymorphic loci

DNA isolated from the individual collected from the river Satluj was digested and after primary screening with prepared repeat motifs amplified with SAULA primer. Cloned inserts after transformation with DH5α cells about 5539 colonies containing both 1444 blue and 4095 white colonies produced and after secondary screening with DIG labeled probes total 226 positive signals were detected on nylon membrane for all types of repeat. After

sequencing of 190 recombinant plasmids 45 repeat regions were isolated for different types of repeat motifs. Dinucleotde shared maximum 55.5% while trinucleotide 6.6%, tetra 13.3%, and mixed 24.4% obtained to the total repeat isolated. Based on sequence quality and flanking length available 29 pairs of primer were designed for 27 repeat sequences. Out of 29 primer pairs tested, 8 polymorphic, 14 monomorhpic loci identified while 7 primers resulted in unspecific multiple product.

Eight microsatellite loci found polymorphic at both $P_{(0.95)}$ and $P_{(0.99)}$ criteria. Allele frequencies ranged from 0.0000 to 0.9333 for different locus in different population. Alleles obtained in the range of two (Cch13) to thirteen (Cch15) and the minimum size obtained at locus Cch6 (87 bp) while maximum size allele found at Cch1 (250 bp). The mean number of alleles per locus in Satluj, Bhagirathi and Brahmaputra were 4.50, 4.87 and 5.25 respectively.

6.2 Genetic variability and population structure of *Chitala chitala*

Based on the allele frequency data various genetic parameters tested in *C. chitala*. Observed heterozygosity ranged from 0.0000 (Bhagirathi and Brahmaputra samples, locus Cch13) to 0.8571 (Bhagirathi samples, locus Cch10) while the expected heterozygosity ranged from 0.1244 (Brahmaputra samples, locus Cch13) to 0.8571 (Bhagirathi samples, locus Cch15). Significant deviation in Hardy-Weinberg equilibrium expectation was evident in Bhagirathi and Brahmaputra samples at locus Cch9 was associated with deficiency of heterozygote and the null alleles may be cause of concern that give rise deficiency of heterozygote. Test for genotypic disequilibrium confirmed that no value was statistically significant ($P>0.05$) for the pair of loci in any samples and across all the samples. This indicated that genotypes are independent and not linked at these microsatellite loci for *C. chitala.*

Genetic differentiation was observed among *C. chitala* populations. When considered for each locus, Bhagirathi and Brahmaputra samples were significant

heterogeneity value to Satluj samples at Cch1 locus. Brahmaputra samples had significant differences with Satluj samples at Cch10 and highly significant value observed between Brahmaputra and Satluj at locus Cch15. Probability values for each population pair across all loci based on Fisher's exact test indicates that four population pairs had significant values after sequential Bonferroni correction ($P<0.002$).

The combined F_{ST} value 0.052405 for all loci in all three populations indicated genetic divergence present between three rivers. Satluj samples are significantly divergent from that of Brahmaputra and Bhagirathi ($F_{ST} = 0.089690$ and 0.048753 respectively) while Brahmaputra and Bhagirathi ($F_{ST} = 0.009773$) showed lower level of divergence.

The study provided information on the genetic variation parameters at microsatellite loci will be useful in planning of the conservation and management strategies of *C. chitala* population in India and makes way for future research on exploring more and better understanding of genetic divergence in *C. chitala.*

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Appendix

APPENDIX

I. Reagents required

1. 0.5 M Tris-HCl (pH 8.0)

Tris base - 3.028 g

Distilled water - 40.0 ml

Adjust the pH to 8.0 using 1N HCl

Makeup final volume to 50ml

Store at 4°C.

2. 0.5 M Tris-HCl (pH 8.3)

Tris base - 3.028 g

Distilled water - 40 ml

Adjust the pH to 8.3 using 1N HCl

Makeup final volume to 50ml

Store at 4°C.

3. 0..5 M EDTA (pH 8.0)

Na_2 EDTA. $2H_2O$ - 9.34 g

Distilled water - 40 ml

Adjust the pH to 8.0 using 0.5M NaOH.

Makeup final volume to 50ml

Store at room temperature.

4. 0.5 M Tris-HCl (pH 7.5):

Tris base - 3.028 g

Distilled water - 40 ml

Adjust the pH to 7.5using 1N HCl

Makeup final volume to 50 ml

Store at 4°C.

5. 5X TAE:

Tris base	-	12.10 g
0.5 M Na_2 EDTA. $2H_2O$ (pH 8.0)	-	5.0 ml
Glacial Acetic acid	-	2.85 ml

Make up the solution to 500 ml with distilled water

Store at room temperature.

6. 0.5X TAE (Gel running buffer):

5 X TAE (stock)	-	25 ml
Distilled water	-	225 ml

Make fresh every time it is required.

7. Bromophenol Blue dye:

Bromophenol blue	-	2.5 mg
Sucrose	-	40.0 mg

Dissolve in 1 ml distilled water

Autoclave.

Store at 4°C.

II. Reagents required for genomic DNA isolation

1. High TE :

Stock 0.5 M Tris-HCl (pH 8.0)	-	20 ml
Stock 0.5 M Na_2 EDTA. $2H_2O$ (pH 8.0)	-	8 ml

Makeup the solution to 100 ml with distilled water

Autoclave it

Cool it down to room temperature

Store at 4°C.

2. Lysis Buffer

Stock 0.5 M Tris-HCl (pH 8.3)	-	2 ml
Stock 0.5 M Na_2 EDTA. $2H_2O$ (pH 8.0)	-	0.2 ml
NaCl	-	2.337 g

Make up the solution to 100 ml with distilled water

Autoclave it

Cool it down to room temperature

Store at 4°C.

3. Proteinase K

Proteinase K	-	10 mg
Autoclaved distilled water	-	500 µl

Dissolve Proteinase K in autoclaved distilled water.

Store at -20°C.

4. RNAase Buffer

0.5M Tris-HCl (pH 7.5)	-	0.2 ml
NaCl (0.292 g in 10 ml)	-	0.3 ml
Distilled water		9.5 ml

Autoclave it

Cool it down to room temperature

Store at 4°C.

5. RNAase

RNAase	-	10 mg
RNAase buffer (autoclaved)	-	1 ml

Dissolve RNAase in RNAase buffer.

Keep the tube in boiling water for 15 minutes.

Allow to cool at room temperature

Store at -20°C.

6. Saturation of Phenol with Tris-HCl (pH 8.0)

6.1 Reagents required

Water saturated Phenol	-	500 ml
0.5 M Tris-HCl (pH 8.0)	-	1000 ml
(60.56 g of Tris base in1000 ml)		
0.1 M Tris-HCl (pH8.0)	-	1500ml

(For 300 ml of 0.5M Tris-HCl (pH8.0) add 1300 ml of Distilled water).

6.2 Procedure

- Add 0.1% 8-hydroxyquinoline to 500 ml of water saturated phenol.
- Cover flask containing phenol with aluminium foil to avoid light reaction.
- Add 500 ml 0.5 M Tris-HCl.
- Stir solution using magnetic stirrer for 15 minutes.
- Keep the solution for 30 minutes to allow phenol to settle.
- Decant the supernatant.
- Add 500 ml of 0.1 M Tris-HCl.
- Repeat the steps of stirring, settling and decanting twice with 0.1 M Tris-HCl..
- Check pH of decanted supernatant using pH paper.
- The final pH should be 8.0.
- Add 500 ml of 0.1 M Tris-HCl to phenol.
- Store at 4°C in dark bottles covered with aluminium foil.

7. Chloroform: Isoamyl alcohol (24:1 V/V)

Chloroform	-	96 ml
Isoamyl alcohol	-	4 ml

8. 3M Sodium acetate (pH 5.2)

Sodium acetate - 12.4 g

Distilled water - 20ml

Adjust the pH to 5.2 using glacial acetic acid

Makeup final volume to 50 ml

Autoclave it

Cool it down to room temperature

Store at 4°C.

9. TE buffer:

Stock 0.5 M Tris-HCl (pH 8.0) - 2.0 ml

Stock 0.5 M Na_2 EDTA.$2H_2O$ (pH 8.0)- 0.02 ml

Make up the solution to 100 ml with distilled water.

Autoclave it

Cool it down to room temperature

Store at 4°C.

III. Reagent for colony lysis on membrane

1. 10%SDS

SDS - 1.0 g

DDW - 10 ml

2. Denaturing solution

Sodium hydroxide - 2.0 g

Sodium chloride - 8.76 g

Dissolve in 100 ml DDW and store at 4°C.

3. Neutralizing solution

Sodium Chloride - 8.76 g

1M Tris-HCl (pH 7.4) - 50.0 ml

4. 2X SSC

10X SSC	-	20 ml
DDW	-	80 ml

IV. Reagent for hybridization and DIG detection

IV.I. Hybridization buffers

1. Strandard hybridization buffer

N-Lauryl sarcosine	-	0.1 g
SDS	-	0.02 g
Blocking reagent (Roche kit)	-	1.0 g
10X SSC	-	50 ml

Make up final volume 100 ml in DDW.

2. 5X SSC

SSC (10X)	-	50 ml
DDW	-	50 ml

3. 0.1X SSC

SSC (10X)	-	1 ml
DDW	-	99 ml

4. 1X SSC

SSC (10X)	-	10 ml
DDW	-	90 ml

IV.II. Reagent for DIG detection

IV.II.1 Stock solution

1. 0.1M Maleic acid buffer

Maleic acid	-	11.06 g
Sodium chloride	-	8.76 g

Dissolve in 700 ml of DDW and set pH 7.5 with solid sodium hydroxide.

2. Blocking solution (10X)

Blocking reagent	-	10 g
Maleic acid buffer	-	100 ml

Dissolve by constant stirring on a heating stirrer with magnet at 65°C.Autoclave and store at 4°C.

3. Detection buffer

1M Tris-HCl (pH 9.5)	-	10 ml
NaCl	-	0.584 g
$MgCl_2.6H_2O$	-	1.01g
DDW	-	90 ml

4. Sodium Citrate (10X SSC)

Sodium citrate	-	44.1 g
NaCl	-	87.65 g

Dissolve in 600 ml DDW set pH 7.0 and make upto1000 ml.

IV.II.2 working solution for DIG detection

1. Washing buffer

Maleic acid buffer	-	485 ml
Twin-20	-	15 ml

Make upto 500 ml in DDW.

2. Blocking solution (1X)

Maleic acid buffer	-	180 ml
Blocking solution (10X)	-	20 ml

3. Color Substrate solution

NBT/BCIP (kit stock)	-	0.2 ml
Detection buffer	-	10 ml

4. Antibody conjugate solution

Antibody conjugate (kit stock)	-	1.0 µl
Blocking solution (1X)	-	10 ml

V. Reagent for bacterial growth

1. 2X YT media

Tryptone	-	1.6 g
Yeast extract	-	1.0 g
NaCl	-	0.5 g
Agar Agar	-	1.5 g

Dissolve in 100 ml DDW, set pH 7.0 and autoclave at 15 psi for 15minutes.Store at 4°C.

2. X-gal solution

20 mg X-gal powder dissolve in 1 ml dimethyl formamide. Sterlize by filter and store at -20°C. Add 40 µl per plate.

3. IPTG solution

200 mg IPTG powder dissolve in 1 ml DDW, sterilize by filter and store in -20°C. Add 4 µl per plate.

4. Ampicillin solution

100 mg ampicilin powder dissolve in 1 ml DDW, sterilize by filter and store in -20°C. Add 1 µl per ml of medium.

VI. Reagent for plasmid isolation

1. Solution-I

Glucose	-	0.9 g
0.5M Tris-HCl (pH 8.0)	-	5.0 ml
0.5M EDTA (pH 8.0)	-	2.0 ml

Make up final volume in 100 ml DDW and store at 4°C.

2. STE buffer

Sodium chloride	-	1.16 g
0.5M Tris-HCl (pH 8.0)	-	4.0 ml
0.5M EDTA (pH 8.0)	-	0.4 ml

Make up final volume in 100 ml DDW and store at 4°C.

3. Solution-II

2N sodium hydroxide	-	1.0 ml
SDS	-	0.1 g

Make up final volume in 10 ml DDW and use fresh condition.

4. Solution-III

Potassium acetate	-	56.4 g
Glacial acetic acid	-	11.5 ml

Make up final volume in 100 ml DDW and store at 4°C.

VII. Reagent required for PAGE

1. 20% Acrylamide

Acrylamide	-	1.9 g
Bis-acrylamide	-	0.1 g

Makeup the final volume to 10 ml with distilled water.

2. 10% Ammonium persulphate

APS	-	100 mg

Makeup the final volume to 1 ml with distilled water.

3. 5X Tris Boric EDTA

Trisbase	-	27 g
Boric acid	-	13.7 g
0.5 M EDTA	-	12.5 ml

Makeup the final volume to 500 ml with distilled water.

4. 1X Tris Boric EDTA

5X TBE	-	60 ml
DDW	-	240 ml

5. Agarose (For sealing of 2 gels)

50 mg / 5 ml (for one set of 2 gel).

6. Different concentrations of polyacrylamide gels for microsatellites study.

Component	**6%**	**8%**	**10%**	**12%**
Acrylamide (20%)	15 ml	20 ml	25 ml	30 ml
DDW	21.5 ml	16.5 ml	11.5 ml	6.5 ml
5X TBE	10 ml	10 ml	10 ml	10 ml
APS (10%)	350 µl	350 µl	350 µl	350 µl
TEMED	17.5 µl	17.5 µl	17.5 µl	17.5 µl

Publications

LIST OF PUBLICATIONS

S. No	Title
1	Polymorphic microsatellite markers isolated from partially enriched genomic library of *Chitala chitala.*

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RIMER NOTE

'olymorphic microsatellite markers isolated from partially :nriched genomic library of *Chitala chitala*

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Abstract

A total eight polymorphic microsatellite loci were obtained from genomic library of Indian feather back, *Chitala chitala* (order Osteoglossiformes, family Notopteridae) and the 46 samples were analysed to determine genetic variation. The mean number of alleles per locus ranged from 4.50 to 5.25, and expected heterozygosity ranged from 0.124 to 0.852. Deviation from Hardy–Weinberg equilibrium expectations ($P < 0.002$) was observed at loci *Cch2*, *Cch9* (Bhaghirathi) and *Cch9* (Brahmaputra). The identified microsatellite loci were found promising for population genetics studies of *C. chitala* and related species *Notopterus notopterus* (family Notopteridae).

Keywords: *Chitala chitala*, microsatellite, *Notopterus notopterus*, null allele, polymorphic, population genetics

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hitala chitala, is widely distributed in freshwater bodies f the Indian subcontinent (Froese & Pauly 2003). *C. chitala* commercially important as ornamental and food fish. 'onservation strategies including propagation-assisted ehabilitation of natural population are necessary in view f the decline in the species' abundance (Sarkar *et al.* 2006). opulation structure of *C. chitala* derived through micro-atellite markers will be useful in planning conserva-on strategies. A microsatellite-enriched genomic library vas constructed following the method of Fleischer & oew (1995) and M. Hamilton and R. Fleischer (personal ommunication). Genomic DNA was extracted from blood nd digested with Sau3Al restriction enzyme. Genomic DNA ragments of 300–800 bp were gel-purified (QUIGEN kit) nd ligated to SAULA (CGGTACCCGGGAAGCTTGG) nd SAULB (ATCCCAAGCTTCCCGGGTACCGC) linkers. 'he fragments were amplified using SAULA as primer in a 0-μL reaction [95 °C, 5 min; 30 × (95 °C, 40 s, 60 °C, 1 min, 2 °C, 1 min); 72 °C, 5 min].

Nylon membranes (5 mm²) saturated with CA/GT, GA/CT and CAGA/GTCT target repeats were hybridized overnight to amplified DNA fragment in DIG Easy Hyb solution (Roche) at 50 °C for dinucleotide and 60 °C for tetranucleotide repeats. The membranes were washed to remove unbound DNA and the hybridized DNA was recovered from the membrane using 100 μL 50 mM KOH/0.01% SDS, followed by 100 μL 50 mM Tris-HCl pH 7.5/0.01% SDS. The recovered DNA was again polymerase chain reaction (PCR)-amplified using SAULA primer and digested with Sau3Al to remove linkers. The DNA fragments were ligated to *Bam*HI digested pUC18 vector and transformed into DH5α competent cells (Invitrogen). Inserts were sequenced using the Big Dye terminator kit with M13 forward primer (MegaBACE, Amersam Biosciences).

A total of 40 repeat sequences were obtained and 29 primer pairs were designed (PRIMER SELECT, version 5.07, DNA Star). PCR amplification was performed in 25 μL reaction on thermocycler (PTC 200, MJ Research) as follows: 5 min denaturation at 95 °C; 25 cycles of 30 s at 95 °C; 30 s at specific annealing temperature (Table 1) and 60 s at 72 °C with a final extension of 10 min at 72 °C. The PCR consisted of 50 ng of DNA, 1× PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.01% gelatin), 5 pmol of each primer, 15 mM $MgCl_2$, 2 μL of 2.5 mM dNTPs and 1.5 U *Taq* polymerase (Genei). Amplified products were resolved on polyacrylamide gel followed by silver staining. Out of 29 microsatellite loci amplified, 8 were polymorphic, 14 were monomorphic and 7 yielded unspecified products. Genotype data at each polymorphic microsatellite locus for samples collected from Satluj ($n = 16$), Bhaghirathi ($n = 15$) and Brahmaputra ($n = 15$) rivers were analysed (GENETIX version 4.05, Belkhir

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Table 1 Characteristics of *Chitala chitala* microsatellites including; F (forward primer), R (reverse primer), T_a (annealing temperature), N_a (alleles observed), H_E (expected heterozygosity), H_O (observed heterozygosity), Sat (Satluj), Bha (Bhaghirathi), Bhm (Bhramputra), P (agreement to HW expectations)

Locus	Primer sequence	Core sequence	T_a	River	N_a	Size range (bp)	H_E	H_O	HW (P)	Genic (P) homogeneity
Cch1	F: CGGAGATGAAGAGCAGCAGTA	$(GA)_{19}(GT)_9$	55	Sat	4	216–232	0.428	0.400	0.718	0.0011**
(DQ525389)	R: TGTGTTCCGTGTTTCCTCCTA	$(GA)_8$		Bha	6	220–250	0.785	0.642	0.244	
				Bhm	6	216–238	0.751	0.733	0.194	
Cch2	F: ACCCAAGCCATGTTAAGTGGTC	$(GA)_{19}GTT$	55	Sat	4	202–228	0.551	0.466	0.155	0.5375
(DQ525390)	R: GGCGAGTCCACGATTTCAAG	$(TG)_9$		Bha	5	194–228	0.617	0.333	0.001**	
				Bhm	5	194–228	0.533	0.600	0.650	
Cch4	F: AGAGATTCCAGCCGCACCACT	$(GA)_{29}$	60	Sat	7	177–205	0.724	0.571	0.071	0.3252
(DQ525391)	R: AGACGCAGCGCGAACTATCACAG			Bha	7	177–205	0.804	0.461	0.003*	
				Bhm	7	177–205	0.770	0.500	0.031*	
Cch6	F: ATTTCCAGCTTCTAACCGCACACC	$(CA)_{13}$	57	Sat	3	83–87	0.460	0.466	0.769	0.0229*
(DQ525392)	R: AGTTGGGGATGCCGCTGTC			Bha	3	83–87	0.636	0.538	0.229	
				Bhm	4	77–87	0.637	0.466	0.048*	
Cch9	F: TGGTGTTGGAGTGTGAGTGCTTAG	$(TC)_{12}(AC)_{14}$	60	Sat	3	222–236	0.526	0.333	0.008*	0.0580
(DQ525393)	R: ATATGCAGTGGCACGACAGGT			Bha	3	222–236	0.571	0.200	< 0.001**	
				Bhm	3	222–236	0.660	0.133	0.000**	
Cch10	F: TCGTTATTTCTGACATTCAAGTGC	$(GT)_{21}$	53	Sat	5	114–132	0.742	0.500	0.015*	0.0108*
(DQ525394)	R: TACAAGCTCCATGCACAATTACAA			Bha	6	114–132	0.783	0.857	0.312	
				Bhm	6	114–132	0.762	0.800	0.623	
Cch13	F: AAGGGTACTGATGAGTGAATGAGC	$(CT)_{14}$	55	Sat	2	220–228	0.191	0.214	1.000	0.9347
(DQ525395)	R: TCATAACAGGCTGTTTATTGTCCA			Bha	2	220–228	0.142	0.000	0.040*	
				Bhm	2	220–228	0.124	0.000	0.034*	
Cch15	F: AACACTGAGCGAAAAGCAACA	$(GA)_5[CA(GA)_3]_4$	55	Sat	8	130–198	0.760	0.615	0.370	0.0004**
(DQ525396)	R: GATAAACGGGTGAGAGCAAGTG	$[CA(GA)_2]_3CA$		Bha	7	130–146	0.816	0.714	0.092	
		$(GACA)_{11}$		Bhm	10	138–184	0.852	0.571	0.006*	
Mean				Sat	4.50	—	0.548	0.568	1	0.0001**
over all loci				Bha	4.87	—	0.636	0.672	1	
				Bhm	5.25	—	0.636	0.475	1	

*Significant ($P < 0.05$); **significant after sequential Bonferroni correction ($P < 0.002$); Accession no. given below the locus name.

et al. 1997) to determine parameters of genetic variation (Table 1). Tests for linkage disequilibrium, genotypic differentiation, and Hardy–Weinberg equilibrium were performed using GENEPOP version 3.4 (Raymond & Rousset 1995). The mean number of allele per locus in Satluj, Bhaghirathi and Brahmaputra were 4.50, 4.87 and 5.25, respectively. Expected heterozygosities ranged from 0.124 to 0.852 and observed heterozygosities from 0.00 to 0.857. After sequential Bonferroni correction, linkage disequilibrium was not detected for any pair of loci ($P > 0.2$) in any sample. Nonconformity to Hardy–Weinberg expectations ($P < 0.002$) was observed on loci *Cch2*, *Cch9* (Bhaghirathi) and *Cch9* (Brahmaputra). The observed heterozygosity deficiency (+ F_{IS}) at these loci might be due to the presence of null allele and/or small sample size. The data were corrected for the possible null alleles (software FREENA, CHAPUIS MP and ESTOUP A) and the corrected data did not exhibited heterozygote deficiency. Significant genetic heterogeneity ($P < 0.002$) was evident at loci *Cch1*, *Cch15* and at the overall loci.

Amplification with five individuals of *Notopterus notopterus* revealed that the loci *Cch1*, *Cch2* and the two loci found monomorphic in *C. chitala* (*Cch18* and *Cch20*) were polymorphic in *notopterus* (Table 2) at annealing temperature of 55 °C. In conclusion, the study identified the microsatellite loci that can be useful in population studies of *C. chitala* and

Table 2 Cross-amplification of primers developed from library of *Chitala chitala* in *Notopterus notopterus*

Locus	Polymorphic	Size range	No. of alleles
Cch1	Yes	180–182	2
Cch2	Yes	216–230	4
Cch4	No	147	1
Cch18	Yes	130–152	4
Cch20	Yes	180–184	2

Cch18 (Accession no. DQ525397; primer forward TGTAGGAGCCGGAGTCGGAGAA, reverse CTGCAGCAGTAGGCCTGTGAGTG).
Cch20 (Accession no. DQ525298; primer forward GGAGGGATGCTGTGCACTATAAAG, reverse CCGTTGGGTCTGTCTGTATATCTG).

N. notopterus; however, cautious interpretation is suggested due to possible presence of null alleles at some of the loci.

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